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A *Bacillus thuringiensis* Insecticidal Crystal Protein with a High Activity against Members of the Family Noctuidae

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The full characterization of a novel insecticidal crystal protein, named Cry9Ca1 according to the revised nomenclature for Cry proteins, from *Bacillus thuringiensis* serovar tolworthi is reported. The crystal protein has 1,157 amino acids and a molecular mass of 129.8 kDa. It has the typical features of the Lepidoptera-active crystal proteins such as five conserved sequence blocks. Also, it is truncated upon trypsin digestion to a toxic fragment of 68.7 kDa by removal of 43 amino acids at the N terminus and the complete C-terminal half after conserved sequence block 5. The 68.7-kDa fragment is further degraded to a nontoxic 55-kDa fragment. The crystal protein has a fairly broad spectrum of activity against lepidopteran insects, including members of the families Pyralidae, Plutellidae, Sphingidae, and Noctuidae. A 50% lethal concentration of less than 100 ng/cm^2 of diet agar was found for diamondback moth, European corn borer, cotton bollworm, and beet armyworm. It is the first insecticidal crystal protein with activity against cutworms. No activity was observed against some beetles, such as Colorado potato beetle. The protein recognizes a receptor different from that recognized by Cry1Ab5 in *Ostrinia nubilalis* and *Plutella xylostella*. In *Spodoptera exigua* and *P. xylostella*, it binds to a receptor which is also recognized by Cry1Cax but with a lower affinity. In these insects, Cry1Cax probably binds with a higher affinity to an additional receptor which is not recognized by Cry9Ca1. Elimination of a trypsin cleavage site which is responsible for the degradation to a nontoxic fragment did result in protease resistance but not in increased toxicity against *O. nubilalis*.

The major characteristic of the gram-positive, sporeforming bacterium *Bacillus thuringiensis* is the production of insecticidal crystal proteins (ICPs) during sporulation. To date, nearly 100 distinct crystal protein gene sequences have been published either in the general scientific literature (5, 8) or in patent applications. These crystal proteins have a specific toxic activity against certain lepidopteran, dipteran, or coleopteran larvae.

Several of the anti-Lepidoptera ICPs have a very high toxic activity against the early-instar larvae of agronomically important pest insects such as *Heliothis* and *Helicoverpa* spp. (budworms and bollworms), *Spodoptera* spp. (armyworms), *Ostrinia nubilalis* (European corn borer), and *Plutella xylostella* (diamondback moth) (12). However, some species of the family Noctuidae such as *Spodoptera frugiperda* and *Agrotis* spp. (cutworms) are insensitive to ICPs. Therefore, we have screened a subset of our collection of 12,000 *B. thuringiensis* isolates for activity against these members of the Noctuidae family. This screening program resulted in the discovery of a novel crystal protein with broad-spectrum activity against members of the Lepidoptera, including several species of the Noctuidae family and some ICP-resistant insects.

Here, we report the full characterization of this novel insecticidal crystal protein, including its gene and protein sequences, its homology with other crystal proteins, its activity spectrum, and the receptor-binding data. In addition, we describe the effect of elimination of a trypsin cleavage site on the activity of the crystal protein.

(The crystal protein, previously named CryIH, was described in a patent application [9]. Preliminary findings were communicated at the Seventh International Conference on Bacillus [1993], Institut Pasteur, Paris, France, and the XXVIth Annual Meeting of the Society for Invertebrate Pathology, Asheville, N.C. [1993].)

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MATERIALS AND METHODS

Isolation of *B. thuringiensis* BTS02618A. *B. thuringiensis* BTS02618A was isolated as described previously by Travers et al. (11) by selective sample enrichment in a buffer containing sodium acetate followed by heat treatment and then plating out on agar plates. The BTS02618A strain reported here was isolated from grain dust collected in Cadlan, Bicol, The Philippines. The strain was deposited at the BCCM-LMG (Belgian Coordinated Collections of Microorganisms-Collection Laboratorium voor Microbiologie, R.U.G., Ghent, Belgium) under accession number P-12593.

Serotyping. Strain BTS02618A belongs to serovar H9 (tolworthi) as determined by J. F. Charles, Institut Pasteur, Paris, France.

ICP gene cloning and sequencing. Total DNA of BTS02618A was partially digested with *Sau*3A and size fractionated on a sucrose gradient. Fragments of DNA between 7 and 10 kb were ligated to *Bam*HI-digested and dephosphorylated pUC19 cloning vector (16). The recombinant plasmids were transformed into *Escherichia coli* MC1061 cells by electroporation. Transformed *E. coli* cells were grown on agar plates (containing 100 μg of ampicillin per ml), and about 10,000 clones were screened by Southern blot analysis with a mixture of nonradioactive digoxigenin-labeled *cry-1*-specific synthetic probes with the sequences 5′TTTTCATCCAGACAAAATT3′, 5′TTCATCAAGATAGAATTCA3′, 5′TTGGATTCGTATTAGTAAA3′, 5′TCACATTTGTTTTAATCC3′, 5′TCTATTGTCAATCGAATTT3′, and 5′TTCTGTACTATTGATTGTA3′. Probe labeling and colony hybridization were carried out as described by the manufacturer (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany). A total of 31 positive clones were selected and analyzed by Southern blot analysis with *Alu*I, using the same set of probes to identify clones carrying the novel *cry* gene. One clone which carried a fragment with the full-length crystal protein gene was sequenced by the chemical modification method of Maxam and Gilbert (10).

Alignment of Cry9Ca1 with other crystal proteins. The amino acid sequence of Cry9Ca1 was deduced from the nucleotide sequence and aligned with the sequences of 43 crystal proteins (Table 1). The alignment was done with the Ifind program (IntelliGenetics, Mountain View, Calif.) with a window size of 10 amino acids and a word length of 2 residues. This program uses the Wilbur and Lipman similarity routine for the alignment. The percent amino acid identity was calculated by dividing the number of matches by the number of amino acid residues in the shorter of the two aligned sequences. This was done for the putative toxic

TABLE 1. Crystal proteins used for amino acid identity determination[a]

ICP	Accession no.	ICP	Accession no.	ICP	Accession no.	ICP	Accession no.	ICP	Accession no.
Cry1Aa1	M11250	Cry1Db1	Z22511	Cry2Ac1	X57252	Cry6Ba1	L07024	Cry12Aa1	L07027
Cry1Ab3	M15271	Cry1Ea1	X53985	Cry3Aa1	M22472	Cry7Aa1	M64478	Cry13Aa1	L07023
Cry1Ac1	M11068	Cry1Eb1	M73253	Cry3Ba1	X17123	Cry7Ab1	U04367	Cry15Aa1	M76442
Cry1Ad1	M73250	Cry1Fa1	M63897	Cry3Bb1	M89794	Cry8Aa1	U04364		
Cry1Ae1	M65252	Cry1Fa3	Z22512	Cry3Ca1	X59797	Cry8Ba1	U04365		
Cry1Ba1	X06711	Cry1Ga1	Z22510	Cry4Aa1	Y00423	Cry8Ca1	U04366		
Cry1Bb1	L32020	Cry1Ha1	Z22513	Cry4Ba1	X07423	Cry9Aa1	X58120		
Cry1Ca1	X07518	Cry1Ia1	X62821	Cry5Aa1	L07025	Cry9Ba1	X75019		
Cry1Cb1	M97880	Cry2Aa1	M31738	Cry5Ab1	L07026	Cry10Aa1	M12662		
Cry1Da1	X54160	Cry2Ab1	M23724	Cry6Aa1	L07022	Cry11Aa1	M31737		

[a] Crystal proteins are named according to the revised nomenclature (5).

fragments (from residue 1 to the C-terminal residue of the fifth conserved sequence block) and for the C-terminal fragments (from the first amino acid after the C terminus of the fifth conserved sequence block to the last amino acid). In cases when no typical conserved sequence block 5 was found (Cry2Aa1, Cry2Ab1, Cry2Ac1, Cry4Aa1, Cry6Aa1, Cry6Ba1, Cry9Ba1, and Cry11Aa1), the whole sequence was included in both alignments. In cases when no typical C-terminal half was found after the conserved sequence block 5 (Cry5A′s, Cry3A1, Cry3Ba1, Cry3Bb1, Cry3Ca1, and Cry10Aa1), only the putative toxic fragments were included in the alignment.

Preparation and purification of crystal protein toxin fragment. A preculture was started by inoculating a 100-ml Erlenmeyer flask containing 10 ml of TB medium with a freshly grown colony of *E. coli* WK6(pHT-NSG1). This medium contained (per liter) 12 g of tryptone (Oxoid), 24 g of yeast extract (Oxoid), 6.3 ml of glycerol, 3.811 g of KH_2PO_4, 12.541 g of K_2HPO_4 (pH 7.1), and 100 μg of ampicillin per ml. Plasmid pHT-NSG1 was constructed by inserting the full *cry9Ca1* operon as an *Eco*RI fragment into the *Eco*RI site of pHT315 (1) behind the *lacz* promotor. The preculture was incubated in an Aqua-Shaker (Adolf Kühner AG, Birsfelden, Switzerland) at 28°C with shaking at 180 rpm for 5 h. The main culture was started by inoculating a 2-liter Erlenmeyer flask containing 200 ml of TB medium and ampicillin with 4 ml of preculture. This main culture was incubated at 25°C with shaking at 120 rpm. Gene expression was induced with isopropyl-β-D-thiogalactoside (IPTG) (final concentration, 1 mM) when the optical density at 600 nm exceeded 0.5 (after approximately 3 h). The induced culture was further grown for about 16 h. Before harvesting, the optical density of the culture was determined (by measuring the optical density of a 10-fold-diluted culture). This value was further used for determining the volumes of resuspension buffers to be added. Cells were harvested by centrifugation at 27,500 × *g* for 20 min, and the supernatant was decanted. The cells were washed by resuspension in 0.2 volume of phosphate-buffered saline (PBS; Oxoid, Columbia, Md.). The cells were pelleted by centrifugation at 27,500 × *g* for 45 min, and the pellet was resuspended in 1 ml of TES buffer (50 mM Tris · Cl, 50 mM EDTA, 15% sucrose [pH 8.0]) per 0.2 optical density at 600 nm unit. The suspension was frozen overnight at −20°C. After the suspension was thawed, 0.5 mg of lysozyme was added per 0.2 optical density unit. In addition, phenylmethylsulfonyl fluoride was added to a final concentration of 0.1 mM to prevent proteolytic breakdown. The suspension was incubated at room temperature for 30 min, and cells were subsequently broken with a French cell press (SLM Instruments, Inc., Urbana, Ill.). Cellular debris was pelleted by centrifugation at 30,000 × *g* for 45 min. The following procedure was used to remove membrane proteins and lipids. The pellet was resuspended in TTN buffer (20 mM Tris · Cl, 1% Triton X-100, 1 M NaCl [pH 7.2]) and thoroughly mixed to wash the cells. The suspension was recentrifuged at 27,500 × *g* for 40 min. The washing step in TTN buffer was repeated twice. The resulting pellet was further washed twice with PBS-acetone (5:1, vol/vol) and finally washed once with PBS. The washed suspension was centrifuged, and the pellet was recovered. For extraction of the crystal protein, the pellet was suspended in 1 ml of alkaline buffer (50 mM Na_2CO_3, 10 mM dithiothreitol, 5 mM EDTA [pH 10.0]) per initial 0.2 optical density unit. Phenylmethylsulfonyl fluoride was added to a final concentration of 0.1 mM. This mixture was shaken at 4°C for 2 h to solubilize the crystal protein inclusions. The solution was then centrifuged at 27,500 × *g* for 30 min, and the supernatant containing the protoxin was recovered. This protoxin solution was dialyzed against a buffer containing 20 mM Tris · Cl (pH 8.6) and 0.2 M NaCl by using a Spectrapor dialysis membrane (Spectrum Medical Industries Inc., Los Angeles, Calif.) with a cutoff value of 12,000 to 14,000. This material was used to prepare purified protoxin by ion-exchange chromatography.

The toxic fragment was generated by adding trypsin to the protoxin solution (1 mg/20 mg of protoxin) and incubating the mixture at 37°C for approximately 3 h. The degree of truncation was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. After completion of the truncation, the process was stopped by adding 0.1 mM phenylmethylsulfonyl fluoride. This crude toxin solution was redialyzed against a buffer containing 20 mM Tris · Cl (pH 8.6) (without salt). This solution was used for further purification of the toxin by ion-exchange chromatography. A sample of the crude toxin solution was applied to a MonoQ HR10/10 column (Pharmacia Biotech AB, Uppsala, Sweden). The material was eluted from the column in a linear gradient ranging from 0 to 0.6 M NaCl in 30 ml of 20 mM Tris · Cl (pH 8.6). Fractions containing the toxic fragment were identified by SDS-PAGE analysis and bioassays (see below). The toxin concentration was determined by optical density measurement at 280 nm and calculated on the basis of a molar extinction coefficient (ε_M) of 57,983 liter mol^{-1} cm^{-1}. The purified crystal protein solutions (protoxin or toxin) were stored at 4°C until use.

Determination of insecticidal activity spectrum. Toxicity assays were performed with neonatal larvae (except for *P. xylostella*, for which third-instar larvae were used) fed on an artificial diet. The diet was dispensed in Multiwell-24 plates (Corning Costar Corp., Cambridge, Mass.) or, for *Manduca sexta*, in 100-cm^2 square petri dishes (Bibby Sterilin Ltd., Stone, England). A 50-μl volume of each of five appropriate toxin dilutions was applied to the surface of the diet in each 2-cm^2 well (Multiwell-24 plates), or 200 μl was applied in each 4-cm^2 well (Sterilin plates). The plates were then dried in a flow hood. The dilutions were made in a PBS–0.1% bovine serum albumin (BSA) buffer. A total of 24 larvae (2 per well) were used per dilution except for *M. sexta* (20 larvae, 4 per 4-cm^2 well) and *Heliothis* and *Helicoverpa* spp. (20 larvae, 1 per well). The Multiwell plates were covered and placed at 25°C in 60 to 70% humidity with a 16-h-light, 8-h-dark cycle. Mortality was scored after 5 or 6 days, and 50% lethal concentration (LC_{50}) data were calculated by Probit analysis (6).

Receptor-binding experiments. Heterologous competition experiments were performed to study the relation between receptors for Cry9Ca1, Cry1Cax (named Bt15 in reference 14 but not deposited in any database and belonging to the Cry1Ca class of toxins), and Cry1Ab5 in *O. nubilalis*, *Spodoptera exigua*, a susceptible laboratory population of *P. xylostella*, and a Javelin-resistant population of *P. xylostella*. The resistant *P. xylostella* colony originated from Florida and was established from last-instar larvae and pupae collected from fields in Loxahatchee (10a). The colony was exposed to a Dipel crystal-spore preparation every three generations to maintain the resistance. The other insects came from our in-house colonies.

Biotinylation of the crystal protein was performed as follows. Toxin samples of the Cry9Ca1 Ala mutant (consisting of bands A and B [see below]) prepared as described above were first dialyzed against $NaHCO_3$ buffer (100 mM $NaHCO_3$, 150 mM NaCl [pH 9.0]). A 1-mg sample of Cry1Ab5, Cry1Cax, and Cry9Ca1 was mixed with 40, 120, and 280 μl of biotinyl-*N*-hydroxysuccinimide ester (BNHS; Amersham International, Little Chalfont, England), respectively. A higher ratio of label to toxin was used for Cry1Cax and Cry9Ca1 because labeling was less efficient. The mixture was incubated for 1 h at 4°C under constant agitation. Toxin samples were loaded onto a Sephadex G-25 (Pharmacia Biotech AB) column to separate biotinylated toxin from free BNHS. The A_{260} and A_{280} of all fractions were determined. Since free BNHS has a stronger A_{260} than A_{280}, it is possible to use this procedure to identify the fractions containing biotinylated toxin. The concentration of biotinylated toxin in these fractions was derived from the A_{280} readings. Fractions were not pooled. Biotinylated ICPs are referred to as B-ICP (e.g., B-Cry1Ab5).

Brush border membrane vesicles (BBMVs) from *O. nubilalis* and *S. exigua* were prepared as described by Van Rie et al. (14). BBMVs from *P. xylostella* were prepared from whole larvae as follows. Last-instar *P. xylostella* larvae were starved for 1 h and frozen in liquid nitrogen. A total of 20 ml of MET buffer [300 mM mannitol, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 17 mM Tris · Cl (pH 7.5)] was added to 900 frozen resistant larvae. A total of 30 ml of MET buffer was added to 1,500 larvae of the susceptible population. The respective mixtures were blended for two 1-min intervals at high speed with a blender (Waring Products Division, Dynamics Corp. of America, New Hartford, Conn.). One volume of 24 mM $MgCl_2$ was added, and the mixture was left on ice for 15 min. After centrifugation for 15 min at 6,000 × *g* and 4°C, the pellet carrying most of the cuticular fragments was

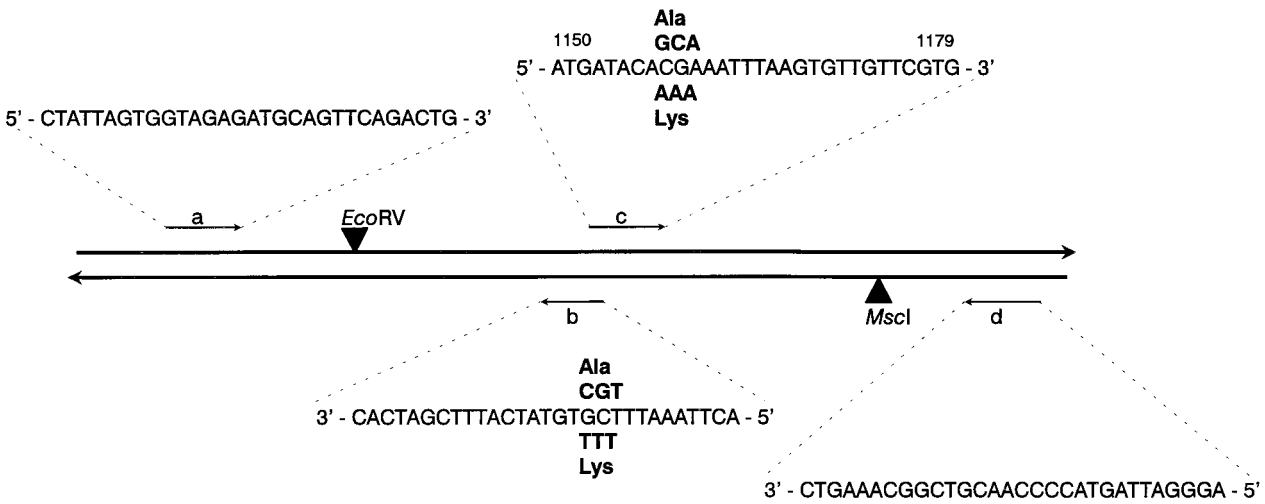

FIG. 1. Elimination of a proteolytic cleavage site by site-directed mutagenesis, using overlap extension. Plasmid pUC-NSG was used for these experiments. Two PCRs were carried out to generate two fragments, ab and cd, which incorporate the mutations. The lines with arrows represent the template DNA and primers in the 5′-to-3′ direction. The sequences of the primers and the positions of the central primers on the full-length sequence are also shown. The mutant codons incorporated in primers b and c and the corresponding amino acids are given in boldface type above and below the original sequence. The triangles show the positions of the restriction sites used for further cloning of the fragments.

discarded. The supernatant was centrifuged at 11,000 × *g* for 30 min at 4°C. The resulting pellet had two layers. The bottom layer had a creamy color and probably contained mainly fatty acids. The upper, greenish layer was removed and resuspended in 0.5 homogenate volume of MET buffer. Another round of mixing, $MgCl_2$ addition, and centrifugation at 6,000 × *g* and 11,000 × *g* was performed, and the resulting pellet was resuspended in half-strength MET solution. Aliquots of this BBMV preparation were stored at −70°C until use. The concentrations of the BBMV preparations were determined by the method of Bradford (3).

Binding assays with B-ICPs on BBMVs were done as follows. An aliquot of BBMVs was thawed and centrifuged in a Biofuge (Heraus Christ GmbH, Osterode, Germany) at 13,000 × *g* at 4°C. The resulting pellet was resuspended in PBS–0.1% BSA to give a final concentration of 1 mg/ml. BBMVs were added to an appropriate amount of biotinylated ICP in PBS–0.1% BSA (in a total volume of 100 μl) and incubated at room temperature for 1 h. The mixture was centrifuged at 13,000 × *g* at 4°C for 10 min. The resulting pellet was washed with 500 μl of PBS–0.1% BSA and finally solubilized in 30 μl of sample buffer mix. Bound ICPs were visualized essentially as described by Bosch et al. (2). The solution was incubated at 95°C for 10 min. Samples were then separated by SDS-PAGE and subsequently blotted on a nitrocellulose membrane. This membrane was incubated overnight in blocking buffer, washed in TBST (20 mM Tris, 0.2% Tween 20 [Sigma, St. Louis, Mo.], 137 mM NaCl [pH 7.6]), and incubated for 30 min with streptavidin-horseradish peroxidase conjugate diluted 1/1,000 in Tris-buffered saline containing 0.1% BSA. The membrane was then washed for 10 min in TBST and for a few minutes in tap water. These washing steps were repeated three times. Excess fluid was removed from the washed membrane, and a mixture of equal volumes of detection solutions 1 and 2 (Amersham RPN 2109) was prepared and applied to the membrane. After a 1-min incubation period, the detection solution was drained off and the membrane was placed on a piece of filter paper (Whatman Int., Ltd., Maidstone, England) for 1 min to remove excess solution fluid. A scientific imaging film (Eastman Kodak Co., Rochester, N.Y.) was exposed for 30 s to the membrane wrapped in plastic foil. Additional exposures were taken after longer exposure times if needed.

Elimination of a proteolytic cleavage site. A trypsin cleavage site at residue 164 of the full-length crystal protein was removed by substituting the Arg residue with Ala. Substitution with Lys was also done because it is not always recognized as a trypsin recognition site (4) and because it is physicochemically (and possibly also functionally) equivalent to Arg. This was accomplished by site-directed mutagenesis via overlap extension by PCR as described by Ho et al. (7). The *cry9Ca1* operon was first cloned as an *Eco*RI fragment in the *Eco*RI site of pUC19, yielding pUC-NSG. A schematic diagram and the details of the procedure used are shown in Fig. 1. The positions of the primers and relevant restriction sites are also shown in Fig. 1. Both fragments were analyzed on an agarose gel containing 2% agarose in TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]). The fragments (ab and cd) were eluted from the agarose gel with a Gene Clean kit (Bio 101, Inc., La Jolla, Calif.). A third reaction was then carried out with a mixture of the two fragments as the template and primers a and d as PCR primers. After the ad fragment was purified, it was digested sequentially with *Eco*RV and *Msc*I. This fragment was ligated to the *Eco*RV- and *Msc*I-digested pUC-NSG plasmid to replace the original fragment with the mutant fragment. The ligation mixture was electroporated into MC1061 cells, and several clones were analyzed by restriction analysis. Positive clones were sequenced to confirm the presence of the mutations. These were used for production of mutant Cry protoxins with Lys or Ala at residue 164.

Nucleotide sequence accession number. The *cry9Ca1* nucleotide sequence accession number at EMBL is Z37527.

RESULTS

Nucleotide and deduced amino acid sequences of the *cry9Ca1* operon. The full sequence of the *cry9Ca1* operon is presented in Fig. 2. The *cry9Ca1* operon consists of two open reading frames (ORFs) preceded by a promoter. The promoter sequence was identified by alignment with upstream sequences of other *cry* sequences and found to be identical to the *cry2Ac1* operon promoter (15). In addition, the protein encoded by the first ORF was identical to the protein encoded by *orf1* of the *cry2Ac1* operon. This protein is 167 amino acids long (or approximately 7 kDa in size). The second ORF encodes an insecticidal crystal protein of 1,157 amino acids (129.8 kDa in size). Putative ribosome-binding sites for both ORFs and an inverted repeat presumably functioning as a transcription terminator are also indicated.

Sequence similarities of Cry9Ca1 with other Cry proteins. The homology between the N-terminal half of Cry9Ca1 and corresponding fragments of the other crystal proteins was generally low and at best was 51% (for Cry1Ba1). Cry8Aa1, Cry1Bb1, and Cry5Aa1 showed homology of 49, 45, and 42%, respectively. All the others were less than 40% homologous. The level of homology of the C-terminal half and corresponding fragments of the other crystal proteins was generally higher. There was a striking 88% homology with Cry9Aa1. Three other crystal proteins, Cry8Aa1, Cry8Ba1, and Cry8Ca1, showed homology of 58, 54, and 55%, respectively.

Insecticidal activity spectrum. The Cry9Ca1 toxin showed a rather broad spectrum of activity against many agronomically important lepidopteran insects. The LC_{50} data of Cry9Ca1 toxin in comparison with the other most efficient ICPs on several lepidopteran insects are presented in Table 2. Above all, it is very toxic for members of the Noctuidae such as *S. exigua*, *S. littoralis*, *Mamestra brassicae*, and *Agrotis segetum*. Of all ICPs tested in our laboratory, it is the only one with activity

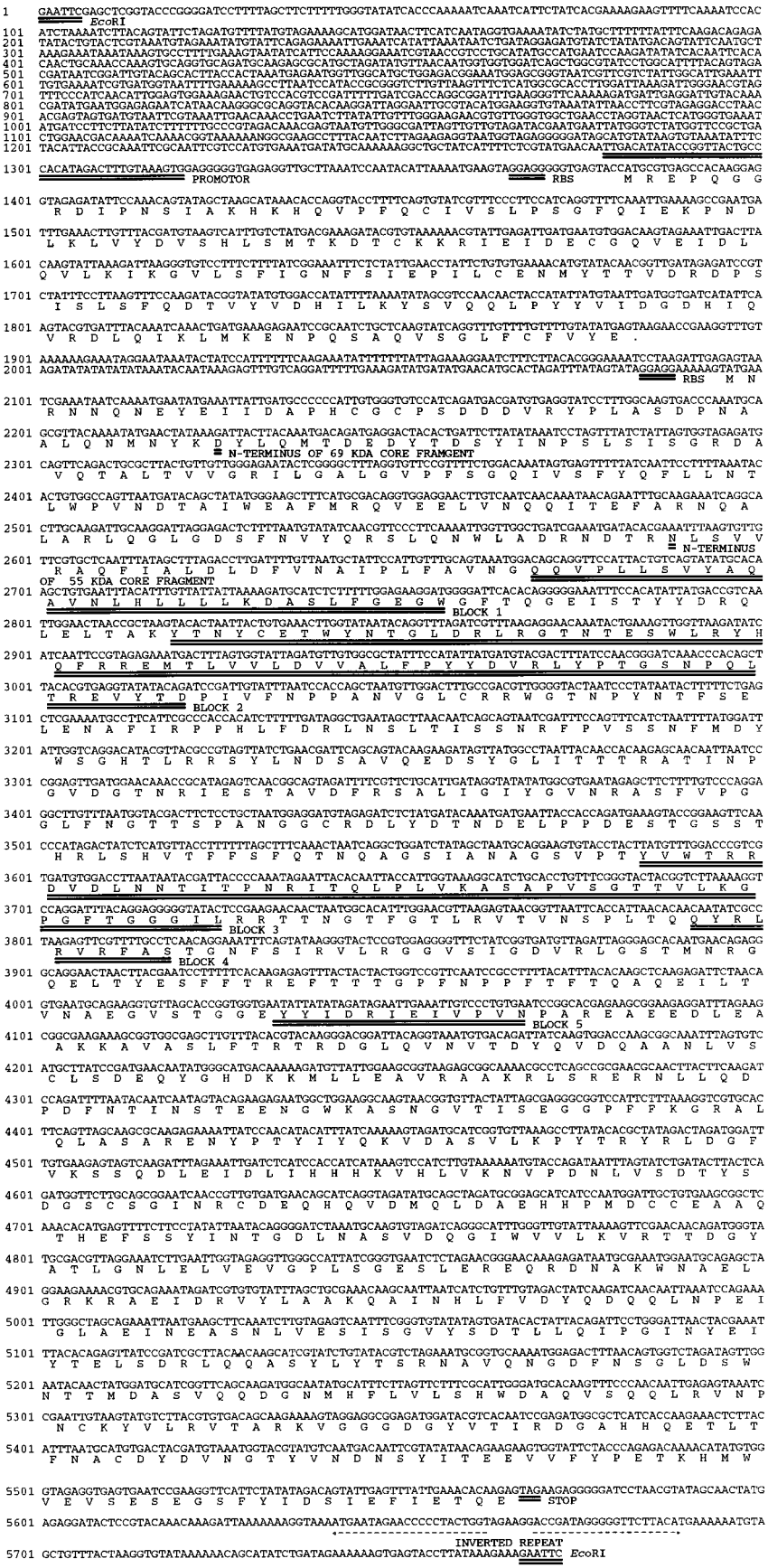

FIG. 2. Nucleotide and deduced amino acid sequence of the *cry9Ca1* operon. Underlined DNA and amino acid sequences indicate relevant features explained in the text. Further details are given in Results and Discussion.

TABLE 2. Insecticidal activity spectrum of Cry9Ca1 toxin

Insect species[a]	Larval stage	CryIH LC_{50}[b]	Fl_{95}min–max[c] (slope)	LC_{50}[b] of other most effective ICP[d]
Helicoverpa armigera	Neonate	>1,350.0		78.8 (Cry1Ab5)
Heliothis virescens	Neonate	51.8	39.2–64.9 (2.1)	1.6 (Cry1Ac1)
Mamestra brassicae	Neonate	78.8	53.5–111.4 (1.6)	22.0 (Cry1Ca2)
Manduca sexta	Neonate	83.0	66.9–103.4 (2.5)	5.4 (Cry1Da1)
Ostrinia nubilalis	Neonate	96.6	77.7–119.8 (2.5)	50.0 (Cry1Ab5)
Plutella xylostella^S	Third instar	6.5	4.9–8.2 (2.3)	1.2 (Cry1Ba1)
Plutella xylostella^R	Third instar	6.1	2.8–11.4 (1.5)	2.3 (Cry1Ba1)
Spodoptera exigua	Neonate	132.9	96.7–180.4 (2.7)	68.9 (Cry1Ca2)
Spodoptera littoralis	Neonate	65.5	44.9–93.7 (2.4)	155.0 (Cry1Ca2)
Spodoptera frugiperda	Neonate	>1,350.0		NT[e]
Agrotis segetum	Neonate	234.2	165.2–327.1 (1.6)	>1,350.0

[a] *Plutella xylostella*^{S} refers to a wild-type population of this insect, while *Plutella xylostella*^{R} is a population which has developed resistance to Cry1Aa, Cry1Ab, and Cry1Ac (10a).
[b] Results are expressed as nanograms of toxin per square centimeter of surface. See also Materials and Methods.
[c] Fl_{95}min–max, 95% confidence limit.
[d] The most effective ICP against the indicated insect as determined in our bioassays.
[e] NT, not tested.

against the last insect. It also shows a considerable activity against *Heliothis virescens* but not against *Helicoverpa armigera*, also members of the Noctuidae. The LC_{50} for *S. frugiperda* is more than 1,350 ng of toxin per cm^2 of diet agar surface. It is nearly as toxic as Cry1Ab5 for *O. nubilalis*, the most potent toxin so far described in the literature. In addition, it is highly toxic for both susceptible and Javelin-resistant diamondback moths.

Receptor-binding characteristics. All experiments were performed with Cry9Ca1 toxin samples consisting of mixtures of bands A and B (see below).

(i) *P. xylostella*. Heterologous competition experiments were performed to study the relation between receptors for Cry9Ca1, Cry1Cax, and Cry1Ab5 in *P. xylostella*. Whereas a 20-fold excess of unlabeled Cry9Ca1 could displace most of the bound labeled Cry9Ca1, only a 500-fold excess of unlabeled Cry1Ab5 was able to displace some of the bound labeled Cry9Ca1 in the susceptible population. These data indicate that these two proteins recognize different receptors. Whereas nearly all labeled Cry9Ca1 could be displaced by a 20-fold excess of unlabeled Cry9Ca1, only part of the labeled Cry9Ca1 could be displaced by a 20-fold excess of Cry1Cax. All labeled Cry9Ca1 was displaced by a 100-fold excess of Cry1Cax. This indicates that Cry1Cax recognizes the same receptor as Cry9Ca1, although with lower affinity. In the reverse experiment, unlabeled Cry9Ca1 could not compete with labeled Cry1Cax (Fig. 3). Such competition would be expected if the two proteins recognized the same binding site. We also compared Cry9Ca1 binding to BBMVs from a sensitive and a Javelin-resistant *P. xylostella* colony. The latter colony is highly resistant to Cry1Ab5 (10b) but is not resistant to Cry9Ca1 (Table 2). Binding experiments demonstrated that Cry1Ab5-binding ability is strongly reduced in the resistant strain. In contrast, Cry9Ca1 binds equally well to vesicles prepared from the sensitive strain and from the resistant strain. These data confirm the existence of two distinct receptors for Cry1Ab5 and Cry9Ca1, as inferred from the competition experiments.

(ii) *O. nubilalis*. A 1,000-fold excess of unlabeled Cry9Ca1 was not able to displace labeled Cry1Ab5. Unlabeled Cry1Ab5 shows only a weak competition for labeled Cry9Ca1. These data indicate that these two proteins recognize different binding sites.

(iii) *S. exigua*. Binding of labeled Cry9Ca1 can be prevented by a 20-fold excess of unlabeled Cry1Cax. Conversely, binding of labeled Cry1Cax could not be prevented by a 500-fold excess of unlabeled Cry9Ca1 (data not shown). Whereas a 20-fold excess of Cry1Cax could displace labeled Cry9Ca1, a 500-fold excess of Cry1Cax was needed to displace most labeled Cry1Cax.

Effect of the elimination of a trypsin cleavage site. Trypsin treatment of Cry9Ca1 protoxin showed that it is broken down initially to a fragment of 68.7 kDa (Fig. 4, band A), which is further processed (Fig. 4, lanes 2, 5, 8, and 11) to a 55-kDa fragment (Fig. 4, band C). Both fragments were purified and tested against *S. exigua*. The 68.7-kDa fragment was toxic at the concentrations tested, while the 55-kDa fragment was not

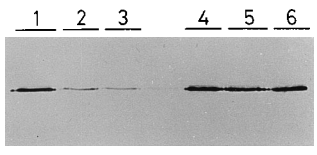

FIG. 3. Binding of biotinylated Cry1Cax on BBMVs of *P. xylostella* with a 20-, 50-, and 500-fold excess of Cry1Cax as a competitor (lanes 1, 2, and 3, respectively) or with a 20-, 50-, and 500-fold excess of Cry9Ca1 (lanes 4, 5, and 6, respectively).

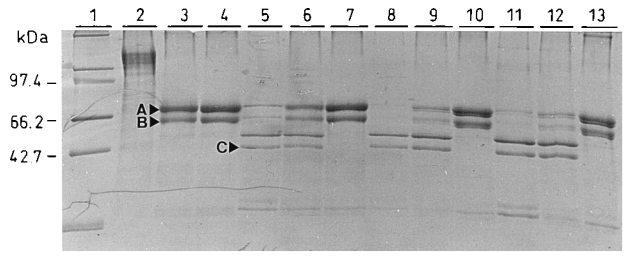

FIG. 4. Trypsinization of wild-type and mutant Cry9Ca1 crystal proteins and visualization by SDS-PAGE. Purified protoxin of the wild-type Cry9Ca1 (lane 2) and the partially digested and purified Lys and Ala mutants (lanes 3 and 4) were treated with 1 μg of trypsin per 20 μg of protein for 3 h (lanes 5 to 7), 20 h (lanes 8 to 10) and 24 h (lanes 11 to 13), respectively. Further batches of trypsin were added after 3 and 20 h. Samples were subjected to SDS-PAGE analysis. The N-terminal sequences of bands A, B, and C were determined and started at positions 44, 58, and 165, respectively, of the Cry9Ca1 protein sequence (see also Fig. 1). Lane 1 contains molecular mass markers.

TABLE 3. Comparative toxicity of wild-type and mutant Cry9Ca1 against *O. nubilalis*

Protein[a]	LC_{50}[b]	Fl_{95}min–max[c]	Slope
Cry9Ca1 protoxin	110.4	57.3–208.6	1.8
Cry9Ca1 (K) protoxin	41.3	33.9–49.8	3.5
Cry9Ca1 (A) protoxin	121.9	100.0–147.6	3.3
Cry9Ca1 (K) toxin	96.3	71.5–129.5	2.8
Cry9Ca1 (A) toxin	64.7	47.1–88.2	2.5

[a] (K) and (A) refer to the lysine and alanine mutants, respectively.
[b] Results are expressed as nanograms of toxin per square centimeter of surface.
[c] Fl_{95}min–max, 95% confidence limit.

toxic. We have attempted to prevent the breakdown of the protoxin or toxin to a nontoxic 55-kDa fragment during in vitro preparation, or possibly during passage through the insect gut, by elimination of the tryptic cleavage site at residues 164 to 165. This was done by substituting the Arg with the neutral amino acid Ala. It was also replaced with the physicochemically similar amino acid Lys.

Partially processed (67- to 69-kDa range) fragments of both mutant protoxins were purified (Fig. 4, lanes 3 and 4) and then tested for resistance to further proteolytic breakdown. It can be observed that only the Ala mutant is not broken down to the nontoxic 55-kDa fragment, even after 24 h of treatment and repeated addition of trypsin (Fig. 4, lanes 4, 7, 10, and 13). After 3 h of trypsin treatment, the protoxin (lane 5) and the Lys mutant (lane 6) are further degraded to fragments ranging from 65 to 50 kDa, with more of the 68.7-kDa fragment remaining in the Lys mutant. In later steps (lanes 8, 9, 11, and 12) the protoxin is more rapidly broken down than is the Lys mutant. Thus, the trypsin cleavage site is effectively eliminated by substitution of Arg at position 164 with Ala. In all samples including the partially digested Ala and Lys mutants (lanes 3 and 4), two major bands can be observed in the 65- to 69-kDa range, each consisting in fact of two bands. Both major bands were N-terminally sequenced. This revealed that the upper band (band A) consisted mainly of a fragment starting at position 44 (Asp) while the lower band (band B) consisted mainly of material starting at position 58 (Ile). N-terminal sequencing of the 55-kDa band indicated that this band starts at position 165. An additional band between the lower band and the 55-kDa fragment was not N terminally sequenced. Purified protoxin of the wild-type crystal protein (lane 2) and both mutants and partially digested and purified fragments of both mutants (lanes 3 and 4) containing the 68.7-kDa fragment (band A) and the 67.1-kDa fragment (band B) were tested against *O. nubilalis*. No significant differences between any of the five preparations were found (Table 3).

DISCUSSION

The novel crystal protein Cry9Ca1 described in this work is not just another crystal protein with a highly toxic activity against lepidopteran larvae. Indeed, its discriminative spectrum of activity makes it one of the most appealing ICPs for the control of agronomically important insect larvae either as sprays or through genetically engineered crop plants. Like some of the commonly used ICPs such as Cry1Ab5 and Cry1Ac1, it shows activity against members of the Pyralidae, the Plutellidae, the Sphingidae, and the Noctuidae (Table 2). Unlike Cry1Ab5 and Cry1Ac1, which are very toxic for *H. armigera* and *H. virescens*, it shows no activity against the former insect and a significant activity against the latter. In our bioassays, it was also very toxic for the diamondback moth, even for a Cry1Ab5-resistant population. Most striking, however, is its high activity against members of the Noctuidae. Like Cry1Ab5 and Cry1Ac1, it is very toxic for the European corn borer. Like Cry1Cax, it displays a strong activity against *S. exigua* and *S. littoralis* but not against *S. frugiperda*. Our bioassay data indicate that is the only ICP with activity against cutworms: it is toxic for the common cutworm (*A. segetum*), and preliminary experiments also indicate an activity against the black cutworm (*A. ipsilon*). In summary, the activity spectrum is most similar to that of Cry1Cax but it is also highly toxic for *H. virescens*, *O. nubilalis*, and *Agrotis* spp. In our hands, Cry1Cax is not toxic for either of these insects.

At the gene level, it is remarkable that the *cry9Ca1* gene is part of an operon. Cry2Aa2 and Cry2Ac1 are the only other examples of crystal protein genes which are organized in an operon (15). These operons contain the ORFs *orf1* and *orf2*, followed by the respective crystal protein genes. The *cry9Ca1* operon consists of two ORFs, the *cry9Ca1* gene and an ORF encoding a protein which is identical (except for one amino acid difference) to the *orf1* product of the *cry2Ac1* operon. The latter is 92% homologous to ORF1 of the *cry2Aa2* operon and appears to be missing 9 amino acids at the N-terminal region. No obvious function for ORF1 or ORF2 in Cry2 protoxin accumulation, solubilization, or toxicity of the crystal proteins was found by Wu et al. (15). We have not attempted to investigate the role of ORF1 in the *cry9Ca1* operon (e.g., by making deletion mutants). However, in analogy with the findings of Wu et al., it seems unlikely that ORF1 would play a crucial role in the activity of the Cry9Ca1 crystal protein.

Competition experiments have shown that Cry9Ca1 recognizes a receptor in *O. nubilalis* and *P. xylostella* different from that recognized by Cry1Ab5. For the latter insect, binding experiments with Cry1Ab5 and Cry9Ca1 on a resistant colony also strongly suggest the presence of two distinct receptors. Competition between Cry9Ca1 and Cry1Cax in both *S. exigua* and *P. xylostella* is somewhat more difficult to interpret. Whereas Cry1Cax can outcompete Cry9Ca1, the reverse could not be demonstrated. Perhaps Cry1Cax recognizes two binding sites, only one of which is also recognized by Cry9Ca1. Cry1Cax may bind preferentially to the receptor not recognized by Cry9Ca1. Competition experiments in which Cry1Cax was used as the competitor to displace labeled Cry1Cax and labeled Cry9Ca1 indicate that Cry1Cax has a higher affinity for the shared receptor than for the additional receptor not recognized by Cry9Ca1. Detailed radioligand-binding experiments would be needed to test this receptor model.

Upon in vitro trypsinization of the wild-type protoxin, toxic 68.7- and 67.1-kDa fragments were generated and were further processed to a nontoxic 55-kDa fragment after longer incubation. N-terminal sequencing of the 55-kDa fragment revealed the presence of a trypsin cleavage site (Arg at position 164). It was hypothesized that the same processing might occur in the larval insect gut by serine proteases, i.e., that when the protoxin was ingested, it would be quickly degraded to a nontoxic fragment. We reasoned that elimination of the trypsin cleavage site at position 164 by substitution with a neutral amino acid such as Ala or with Lys would result in an increased toxicity, since the end product of the proteolysis would be only a toxic fragment and hence a larger amount of toxic material could be expected to accumulate. In a similar experiment, Brinkmann et al. (4) successfully eliminated a protease cleavage target in *Pseudomonas* exotoxin A by replacement of the Arg residue with Lys. This resulted not only in protease resistance and full toxicity but also in a prolongation of the survival of the exotoxin molecule in mice. However, the Lys mutant of Cry9Ca1 was not resistant to trypsin. Probably not only the residue itself

but also the neighboring residues play a role in the recognition of a site by proteases. Trypsinization of the Ala mutant of Cry9Ca1 protoxin showed that it was resistant to proteolytic degradation (Fig. 4), but this did not result in increased toxicity against the European corn borer compared with that of the wild-type protoxin (Table 3). We did not analyze whether there is a significant difference in other insect species. Perhaps in *O. nubilalis* the time frame during which the intermediary protease-sensitive 68.7-kDa fragment is present (at certain concentrations) is sufficient to cause the killing of the larvae. Consequently, elimination of the trypsin site would not increase the activity. The situation might be different in other insect larvae. Indeed, in other poorly sensitive insect larvae (such as *S. frugiperda*), ingestion of a protease-resistant form of the toxin might result in increased toxicity because of the accumulation of the toxic fragment and the exceeding of a minimal threshold value. The Ala mutant of Cry9Ca1 is very valuable for the production of Cry9Ca1 toxin for use in bioassays, mode-of-action studies, etc., because a stable toxic fragment can be generated by simple trypsinization of the mutant protoxin. In fact, when we used the wild-type protoxin, it was difficult to control the processing in such a way that mainly 68.7- to 67.1-kDa material without the contamination of nontoxic 55-kDa fragment was generated. Moreover, because the 55-kDa and other fragments are physicochemically very similar they were very difficult to separate during purification.

In conclusion, Cry9Ca1 is a very valuable ICP because of its broad spectrum of activity and its specific receptor-binding characteristics. It might be of particular value for the control of some corn pest insects since it is toxic to the most important lepidopteran pest, *O. nubilalis*, and a number of secondary pest insects such as armyworms and cutworms. Bioassays on corn plants that have been engineered with Cry9Ca1 have shown that these plants are fully protected against the European corn borer and the common cutworm (unpublished results). Cry9Ca1 toxin binds to other receptors than the crystal proteins that are currently used in sprays or in engineered plants for the control of the Lepidoptera. Since the mechanism of resistance development against these toxins is primarily due to binding-site modification (13), Cry9Ca1 is a valuable tool for resistance management.

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