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CLONING FROM *BACILLUS THURINGIENSIS* SUBSP. *AIZAWAI* 7.29 OF A GENE ENCODING A DELTA-ENDOTOXIN SPECIFICALLY ACTIVE AGAINST LEPIDOPTERAN INSECT SPECIES OF THE NOCTUIDAE FAMILY

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ABSTRACT

Bacillus thuringiensis subsp. *aizawai* 7.29 (HD 137), a strain active against the cotton leaf worm *Spodoptera littoralis* Bdv., harbors at least five della-endotoxin genes: Two of them belong to the *cry IA(b)* class, according to the new classification proposed by Hofte and Whiteley (1989). This type of delta-cndotoxin gene directs, in *E. coli*, the synthesis of a 130 kDa polypeptide which is poorly toxic towards *S. littoralis* but that is preferentially active against the large white cabbage, *Pieris brassicae.* Two other genes *crylC* and *cryID,* of presumed chromosomal origin, belong to new structural types and are located in the same vicinity and transcribed in the same direction. The expression product, in *E. coli,* of the *crylD* gene is a 130-135 kDa crystal protein that is not significantly toxic against the different lepidopteran insect species tested. The product of the *crylC* gene is preferentially toxic to larvae of *S littoralis.* The amino-terminal domain of this polypeptide (amino-acids 1 to 617) is significantly different (55%) from the other lepidopteran-active delta-endotoxins. The similarities (45%) are clustered in five "conserved" domains. These domains are also presentin the dipteran and coleopteran-activc delta-endotoxins, suggesting that they might be functionally important in the mechanism of toxicity.

INTRODUCTION

The insecticidal properties *of Bacillus thuringiensis* are mainly attributed to the parasporal crystalline inclusions synthesized during the sporulation phase (Ribier and Lecadet, 1973). These inclusions are composed of protoxin molecules, designated as delta-endotoxins, that are proteolylically converted to toxic polypeptides by the host gut proteases of the susceptible insect larvae. Genetics studies have shown that the structural toxin genes are generally located on high molecular weight plasmids (Gonzalez et al., 1982). In several cases a chromosomal localization of the genes was also reported (Klier et al., 1982; Sanchis et al., 1988). Furthermore, it has been demonstrated that in some strains, several crystal genes are present at different locations (Kronstad et al., 1983). These observations are consistent with the fact that these genes are frequently associated with transposable elements: Tn4*430* and IS237 (Lereclus et al., 1986; Mahillon et al., 1985). The number of toxin genes in a strain varies from one to at least five (Kronstad and Whiteley, 1986; Sanchis et al., 1988) and several classes of dclta-endotoxin genes have been identified on the basis of structural differences between their products.

Because of the high specificity of the delta-endotoxin gene products, a number of lepidopteran species of agronomical importance, mainly *Heliothis* spp. and *Spodoptera* spp., are poorly suscep-

tible to the commercial isolates of *B. thuringiensis* presently used as pesticides. These insect species are now, in many areas, tolerant or resistant to many chemical pesticides, including pyrethroids. Such is the case for the cotton leaf worm *S. littoralis*, a polyphagous insect, which is a major pest of cotton and other crops of industrial importance (maize, castor-oil plant, tobacco and peanut) and of various fodder plants (clover and alfalfa) or market garden products (cabbage and tomato). Various *B. thuringiensis* strains of the subsp. *aizawai* were characterized by Kalfon and de Barjac (1985) for their significant level of activity against *S. littoralis*. One of these strains, strain *aizawai* 7.29 (HD 137), was chosen as a tool for studying the determinants involved in host range specificity.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacillus thuringiensis subsp. *aizawai* 7.29 used in this study is a natural isolate obtained from the WHO collaborating Center for Entomopathogenic Bacillus (Institut Pasteur); we are grateful to H. de Barjac for providing this strain. *E. coli* strain JM83 was used as a recipient strain for transformation and plasmids pUC8, pUC9 and pUC18 (Vieira and Messing, 1982) were used for cloning experiments.

Nucleic acids procedures

All enzymes were used as recommended by the manufacturers and the basic recombinant DNA procedures were the same as those described by Sanchis et al. (1988).

Electrophoresis and immunoblotting

SDS-acrylamide gel electrophoresis was conducted by the method of O'Farrell (1975) using 10% single concentration gels with an acrylamide/*N,N*-methylene bisacrylamide ratio of 100:1.

Electrotransfer on nitrocellulose membranes followed by immunodetection was done as described by Towbin et al. (1979) with goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase as the second antibody.

Preparation of cell extracts from *E. coli*

E. coli soluble protein extracts from *E. coli* recombinant clones bearing the different types of crystal protein genes from *Bacillus thuringiensis* subsp. *aizawai* 7.29 were prepared as described by Lecadet et al. (1988). The protein extracts were used both for toxicity assays and for the analysis of the expression products of the cloned genes.

Bioassays and insecticidal activity

The toxicity of *E. coli* recombinant clones and of *Bacillus thuringiensis* subsp. *aizawai* 7.29 was estimated using soluble protein extracts from *E. coli* or solubilized purified crystal preparations from *B. thuringiensis*; the toxicity was determined in terms of LD 50 by biological assays using caterpillars at the 5th instar of species *Pieris brassicae* and *Spodoptera littoralis*. Biological assays were conducted as previously described by Lecadet and Martouret (1987) by the forced feeding technique. For estimating the toxicity of the soluble *E. coli* extracts in terms of specific crystal proteins, immunoprecipitation experiments were conducted as previously described by Lecadet et al. (1988) in order to determine the levels of expression of the different cloned genes in *E. coli*. The 50% lethal doses were determined on the basis of an average individual weight of 41- or 113-mg for *S. littoralis* and *P. brassicae*, respectively.

RESULTS

1. Cloning and characterization of different delta-endotoxin genes from *B. thuringiensis* subsp. *aizawai* 7.29

The toxicity of crystal preparations from *B. thuringiensis* subsp. *aizawai* 7.29 and from other more commonly used strains of *B. thuringiensis*, such as *B. thuringiensis* subsp. *thuringiensis* strain berliner 1715 or *B. thuringiensis* subsp. *kurstaki* HD1, was determined in terms of LD 50 values by biological assays using 5th instar larvae of *S. littoralis* and forced feeding techniques (Lecadet and Martouret, 1987). The results indicated that the crystals from *B. thuringiensis* subsp. *aizawai* 7.29 were the most active against *S. littoralis*. The specific toxicities of the crystals originating from these different strains against two insect species, *S. littoralis* and *P. brassicae*, were also determined in terms of LD 50 ratios. The ratio LD 50 *S. littoralis*/LD 50 *P. brassicae* was designated as an index of specificity and used to identify different type activities among natural isolates as well as from *E. coli* cells harboring cloned genes.

DNA-DNA hybridization experiments were performed using as a probe a ^{32}P labeled internal fragment (2 kb) of the *B. thuringiensis* var *thuringiensis* strain berliner 1715 delta-endotoxin gene (Klier et al., 1982). The results (data not shown) indicated that at least five delta-endotoxin genes were present in strain *aizawai* 7.29, one of which is borne by the 45 MDa resident plasmid (pBT45). This plasmid, transferred by mating into a *Bacillus cereus* recipient strain, induced the synthesis of crystalline inclusions that were poorly toxic towards *S. littoralis*, but were preferentially active against *P. brassicae*. SDS-gel electrophoretic analysis indicated that the crystalline inclusions produced by the *B. cereus* transconjugant were constituted of only one polypeptide of about 130 kDa (Fig. 1, lane 2). The electrophoresis study of crystals from *B. thuringiensis* var *aizawai* 7.29 indicated the presence of at least two distinct polypeptides, seen as a doublet band at 130 to 140 kDa (Fig. 1, lane 3); it also appears as indicated in Fig. 1, that the crystal protein gene located on pBT45 encodes a 130 kDa protein that corresponds to the lower band of the doublet.

A 18 kb *Bam*HI DNA fragment, which carries the delta-endotoxin gene located on pBT45, was cloned into the recombinant plasmid pBT45-1 (Klier et al., 1985). The restriction map of this DNA fragment was determined and hybridization experiments were performed using as probes the transposable elements Tn*4430* (Lereclus et al., 1986) and IS*231* (Mahillon et al., 1985). The results, presented in Fig. 2, indicated that the plasmid delta-endotoxin gene belonged to the *cryIA(b)* class of crystal protein genes and was associated with the transposable elements Tn*4430* and IS*231* (as it is the case in many strains for this type of gene). A second delta-endotoxin gene, contained in a 14 kb *Bam*HI DNA fragment isolated from the total DNA preparation of *B. thuringiensis* var *aizawai* 7.29, was cloned in the recombinant plasmid pHTA1 and compared to pBT45-1 (Fig. 2). The results indicated that the only differences identified between pHTA1 and pBT45-1 were in the DNA flanking Tn*4430*, the region of both sides varying in both length and restriction map. Therefore, it appears, that this type of crystal protein gene and its surrounding sequences are duplicated in strain *aizawai* 7.29; one copy is located on a 45 MDa plasmid, whereas the other copy seems to be on the chromosomal DNA or on a large plasmid which is indistinguishable from the chromosome by the usual techniques of DNA preparation. This observation strongly suggests that this type of delta-endotoxin gene is part of a transposon-like structure that could be responsible for its duplication.

A high level of expression in *E. coli* of the pHTA1 crystal protein gene (*cryIA(b)*) was obtained with a pHTA1 derived plasmid designated pHTA2 (figure 2). The *E. coli* cells containing pHTA2 were able to produce genuine crystals that were also preferentially active against *P. brassicae* larvae (Table 1).

A third delta-endotoxin gene, contained on a 6 kb *Bgl*II DNA fragment, and a large part of a fourth gene, contained on a 6.6 kb Pst DNA fragment, were also isolated from the total DNA preparation of strain aizawai 7.29 in plasmids pHTA4 and pHTA6, respectively (Fig. 3). Structural analysis indicated that each gene displayed a unique physical map different from maps already

Fig. 1. Protein analysis of the crystalline inclusions produced by *B. thuringiensis* var *aizawai* 7.29 and *B. cereus* (pBT45). Solubilized purified crystal preparations were analyzed by SDS-PAGE and Coomassie blue staining. Lane 1: molecular weight markers, lane 2: *B. cereus* containing pHT45, lane 3: *B. thuringiensis* var *aizawai* 7.29.

described for the lepidopteran, dipteran and coleopteran toxin genes. These two genes were designated *cryID* and *cryIC*, respectively, according to the new classification proposed by Höfte and Whiteley (1989). These two new genes, of presumed chromosomal origin, were shown to be located in close proximity (3 kb distant) by mapping and hybridization experiments (Sanchis et al., 1988). The direction of transcription of these genes was deduced from the respective positions of the DNA fragments by hybridizing with three specific DNA probes corresponding to the 5′ or 3′ ends of the pHTA2 delta-endotoxin gene (see Fig. 2). The results strongly suggest that these two genes have the same direction of transcription.

A pair of genes apparently homologous to those genes described above were also isolated from the total DNA of *B. thuringiensis* var *entomocidus* 601, a strain which is also very active against *S. littoralis* (Sanchis et al., 1988); The same structural organization was found for these two genes in the *B. thuringiensis* var *entomocidus* 601 strain.

The fifth crystal protein gene identified in strain *aizawai* 7.29 has not been isolated but has been characterized from total DNA and according to its *Pvu*II restriction sites should be related to the *crylA(a)* class (Fig. 3).

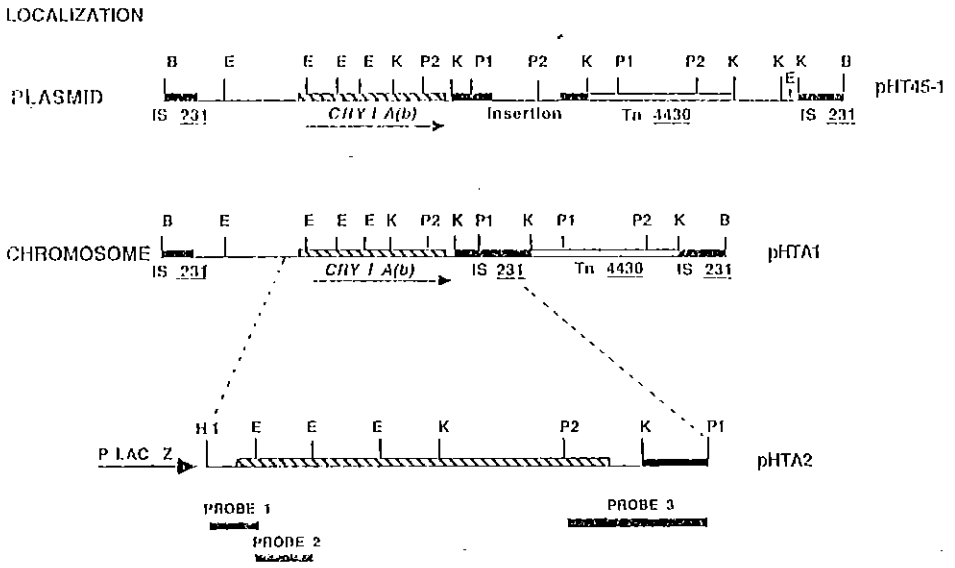

Fig. 2. Restriction map and structural organization of the pBT45-1 and pHTA1 recombinant plasmids harboring the *cryIA(b)* gene and restriction map of the pHTA2. Vectors sequences have been omitted with the exception of the position and direction of transcription of the *lacZ* promoter. The solid bars below the pHTA2 indicate the fragments of the gene used as hybridization probes. The horizontal arrows indicate the direction of transcription of the genes. Abbreviations are: B1: *Bam*HI, B2: *Bg*III, E: *Eco*RI, H1: *Hpa*I, H2: *Hpa*II, K: *Kpn*I, P1: *Pst*I, P2: *Pvu*II, S: *Sst*I.

TABLE 1
Toxicity spectra of the different delta-endotoxins of strain *aizawai* 7.29

Clone	Gene[1]	LD 50[2] on 5th instar larvae		Index of specificity: LD50 *S. littoralis* / LD50 *P. brassicae*	Other target insects
		S. littoralis	*P. brassicae*		
pHTA2	*cry IA(b)*	136	0.24	566	*Bombyx mori* *Manduca sexta*
pHTA4	*cry ID*	NT	NT	–	*Manduca sexta*
pHT71	*cry IC*	3.6	11.8	0.3	*Spodoptera exigua* *Mamestra brassicae*
Strain *aizawai* 7.29 (native crystals)	*cry IA(b)*[3] *cry ID*[3] *cry IC*[3] *cry IA(a)*	2.4	2.15	1.1	*Pieris brassicae* *Spodoptera littoralis* *Spodoptera exigua* *Mamestra brassicae*

[1]According to the classification proposed by H.R. Whiteley and H. Höfte.
[2]LD 50 in micrograms of toxin per gram of larvae.
[3]The gene product was shown to be a component of the crystals.
NT = no toxicity at all.

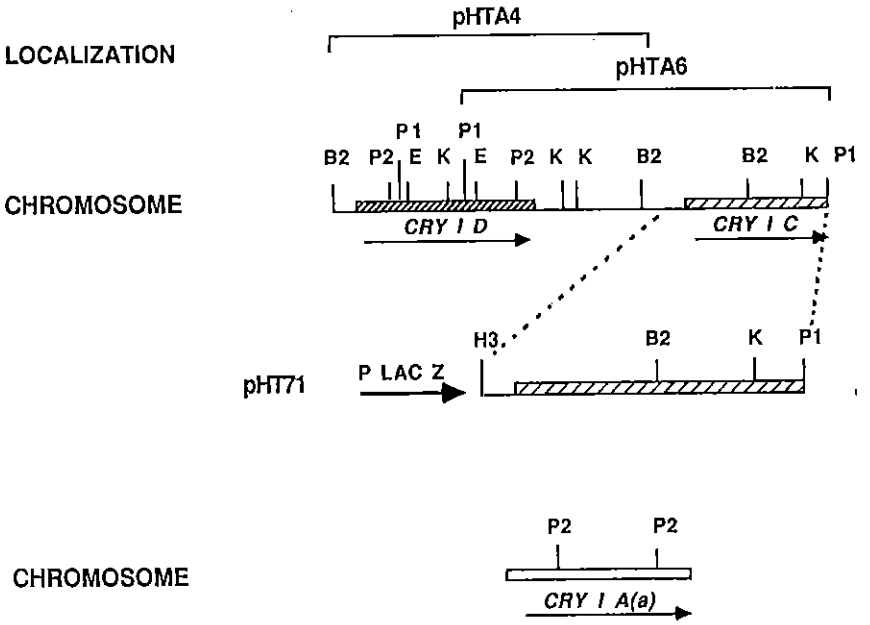

Fig. 3. Physical maps of the two new types of delta-endotoxin genes (*cryID* and *cryIC*) isolated from *B. thuringiensis* var *aizawai* 7.29. Abbreviations are the same as in Fig. 2. Additional abbreviations are: H3: *Hin*dIII.

2. Toxicity spectra of the delta-endotoxin gene products and identification of the gene encoding the *Spodoptera* specific delta-endotoxin

The expression products of the cloned gene in *E. coli* were analyzed by Western blot analysis using polyclonal antibodies directed against the solubilized purified crystals of strain *aizawai* 7.29. Crude protein extracts prepared from *E. coli* cells harboring pHTA4 contained polypeptides specifically recognized by the antibodies indicating that the *cryID* gene is expressed in *E. coli*. Expression of the *cryIA(b)* and *cryIC* genes was obtained when the *cryIA(b)* gene was subcloned in pHTA2 as described above and when the 3 kb *Hin*dIII-*Pst*I fragment which carries the *cryIC* truncated gene was subcloned into pUC 9 to give plasmid pHT71 (Sanchis et al., 1988; 1989).

Crude bacterial extracts or soluble protein extracts prepared from the different *E. coli* recombinant clones were assayed for toxicity against 5th instar larvae of *S. littoralis* and *P. brassicae*. The quantitative estimation of the toxic activity of the different gene products was determined in terms of LD 50 (μg of specific protein per gram of larvae). It must be noted that this is only possible when known amounts of crystal protein are given to each larva; therefore, immunoprecipitation experiments were performed to determine the expression rates of the cloned genes in *E. coli* and the bioassays were performed by the forced feeding technique. The results of the toxicity assays are presented in Table 1.

It appears that the 130 kDa polypeptide encoded by the *cryIA(b)* gene cloned in pHTA2 is toxic to larvae of lepidoptera such as *Bombyx mori*, *Manduca sexta* or *P. brassicae*, but is poorly active against larvae of other lepidoptera such as *S. littoralis* or *Mamestra brassicae*. The toxicity of the *cryIA(b)* gene towards *S. littoralis* and *P. brassicae* was also compared in terms of LD 50 ratio (index

of specificity) and the results indicated that this delta-endotoxin is 500-fold more active against *P. brassicae* than it is against *S. littoralis*.

The product of *cryID* crystal protein gene cloned in plasmid pHTA4 is a 130 kDa polypeptide that is different from the *cryIA(b)* gene product. This crystal protein was not active against the different insect species tested and the target insects of this polypeptide remain to the identified. However, Höfte and Whiteley (1989) reported that this type of delta-endotoxin is active against *M. sexta*.

The 3′ truncated *cryIC* delta-endotoxin gene isolated in plasmid pHT71 determines the synthesis in *E. coli* of a 92 kDa polypeptide highly active against *S. littoralis* and *M. brassicae* (Table 1 and Sanchis et al., 1988) and against *Spodoptera exigua* (Visser et al., 1988). This 92 kDa polypeptide corresponds to a carboxyterminally truncated crystal protein in which the amino-terminal active domain of the protoxin molecule is entirely included (Sanchis et al., 1989).

The results presented in Table 1 clearly indicate that the products of the cloned crystal protein genes differ markedly in their specificity and that the product of the *cryIC* gene is very specific towards *S. littoralis*. Moreover, its LD50 towards *S. littoralis* is of the same order of magnitude as that observed with *B. thuringiensis* var *aizawai* 7.29 native crystals.

3. Amino acid sequence comparison of the *Spodoptera* specific delta-endotoxin with different classes of crystal proteins

The nucleotide sequence of the *cryIC* gene has been determined (Sanchis et al., 1989; Honée et al., 1988). In order to identify conserved features among the different types of crystal proteins whatever their host range specificity, the deduced amino-acid sequence of the CryIC polypeptide was compared to other types of crystal proteins. The amino-acid sequences of the different classes of delta-endotoxins were taken from published data (see legend of Fig. 4 for references) and aligned to the CryIC sequence. Fig. 4 shows the diagram of the amino-acid sequence comparison between different classes of delta-endotoxins; in this diagram the regions represented by different symbols are weakly related, the regions represented by black boxes are highly conserved among the different polypeptides whereas the dashed boxes indicate partial conservation of the amino-acid sequence.

It clearly appears from these comparisons that all the proteins, except the 27 kDa cytolysin of *B. thuringiensis* var *israelensis* (Bourgouin et al., 1986), share common features and structural analogies. The structural analogies suggest an identical ancestral origin for these toxins and that the mode of action of the different toxins might be basically the same.

The 130 kDa polypeptides (CryI and CryIV) active against lepidopteran or dipteran insect species and the 73 kDa polypeptide (CryIIIA) active against coleoptera are the most closely related polypeptides. In the case of the 130 kDa polypeptides it appears that the carboxyterminal moiety is the most highly conserved domain of these crystal proteins. Toxicity experiments carried out with carboxyterminally truncated polypeptides (Adang et al., 1985; Höfte et al., 1986; Schnepf et al., 1985; Delécluse et al., 1989; Sanchis et al., 1989) have clearly shown that this region is not required for toxicity and that the truncated polypeptides retain the toxic specificity of the native delta-endotoxins. Thus, the role of this conserved domain is presumably structural and involved in the formation and stability of the crystal.

The amino-terminal half of these molecules constitutes a variable region that probably reflects the specificity of the toxins; nevertheless, in this variable region several conserved domains are present in all the polypeptides whatever their host range specificity (boxes 1 to 5 of Fig. 4). This suggests an essential function of the conserved domains in the larvicidal activity whereas the variable regions could be responsible for the specificity of the different polypeptides. The products of the *cryIVD* and *cryIIA* genes also clearly belong to this family of proteins but are most distantly related, i.e., the similarities are only found in box 1 and in part in box 2. The *cryIVD* gene product also contains some similarity to box 4 whereas the *cryIIA* gene product contains some similarity to box 5 (fig. 4).

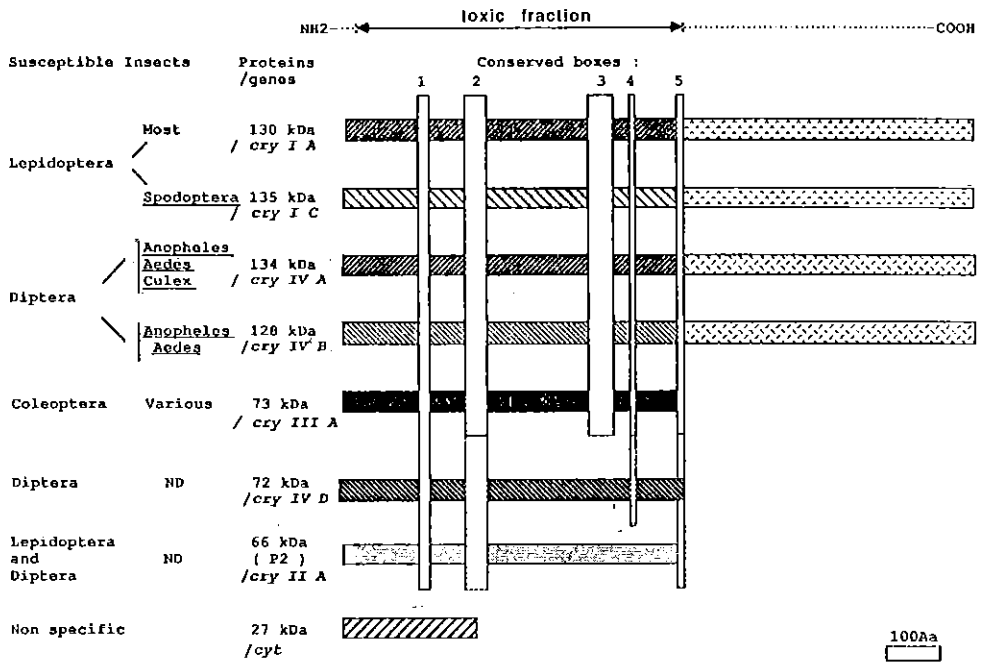

Fig. 4. Amino-acid sequence comparison of the different classes of delta-endotoxin genes. The five regions represented by unshaded boxes are highly conserved among the different proteins. The dotted boxes indicate partial conservation of the amino-acid sequence. In the amino-terminal half of the sequences, the regions represented by diagonally shaded boxes are weakly related. References corresponding to the different genes and proteins are: Schnepf et al., 1985; Honée et al., 1989; Sanchis et al., 1989; Ward and Ellar, 1987; Sen et al., 1988; Hermstadt et al., 1987; Donovan et al., 1988; Widner and Whiteley, 1989; Waalwijk et al., 1985.

These similarities are presented in Fig. 5 which represents the alignment of the five amino-acid sequences conserved among the different toxins. The results indicate that boxes 1 and 2 are well conserved among the different polypeptides; however, it should be mentioned that a greater similarity can be observed between the *cryIVD* and *cryIIA* gene products in the box 2 region than between each of them and the 130 kDa family. The similarities specifically observed between these two gene products have been boxed differently in Fig. 5. Box 3 is also very well conserved but only among the CryIIIA, CryIVA and B and CryI crystal proteins. The *cryIVD* gene product possesses some similarity to box 4 and the repetitive nature of the sequence, which is rich in arginine residues, allows two possible alignments which are shown in Fig. 5. It must be noted that in this case, and in the case of the similarity found between the *cryIIA* gene product and box 5, the similarities are relatively weak but are probably significant since they occur at the expected place along the sequence.

CONCLUSIONS AND PERSPECTIVES

Our results clearly indicate that different delta-endotoxin genes (at least five) are present in *Bacillus thuringiensis* var *aizawai* 7.29 with four of the genes belonging to three different structural types, *cryIA(b)*, *cryIC* and *cryID* isolated by cloning into *E. coli*. Their expression products in *E. coli* display distinct specific activities against different lepidopteran insect species and comparative toxicity

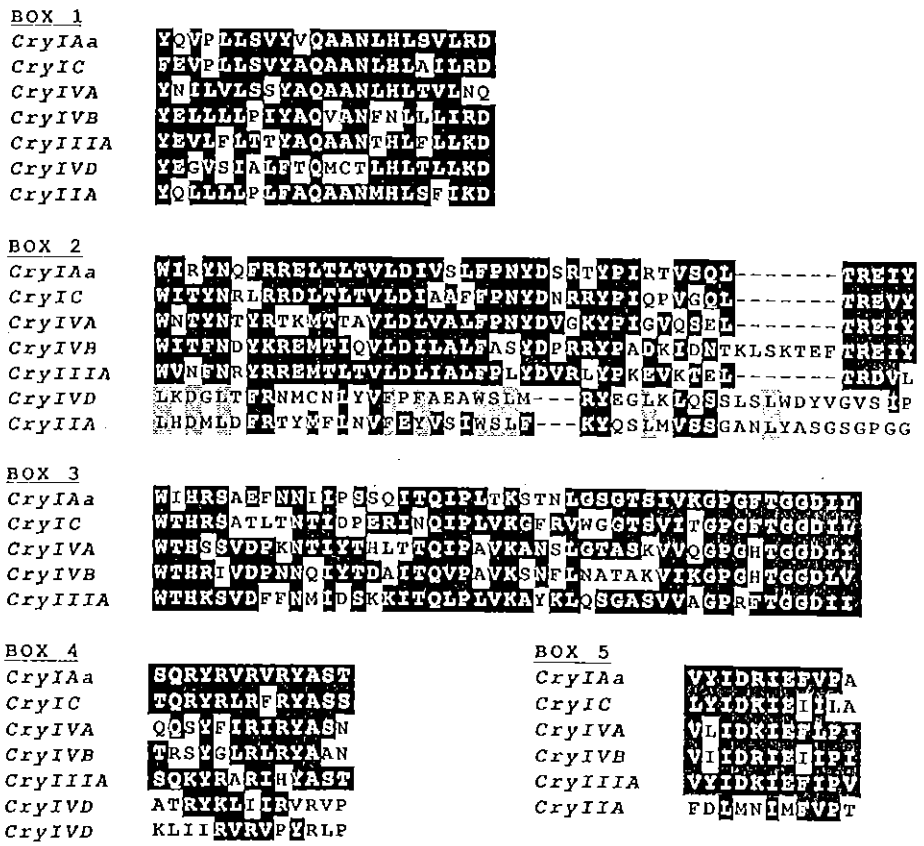

Fig. 5. Alignment of the five amino-acid sequences conserved in the lepidopteran, dipteran and coleopteran active delta-endotoxins. The relative positions of the five conserved boxes, previously described by Sanchis et al. (1989), are indicated in Fig. 4. Identical and similar residues are boxed with black. Accepted conservative replacements used are I, L, V and M; D and E; N and Q; R and K; S and T; F and Y. In box 2 the similarities specifically observed between the CryIVD and CryIIA polypeptides are boxed in grey.

clearly indicate that the product of *cryIC* gene is preferentially toxic to larvae of *S. littoralis* These data strongly suggest that the activity of strain *aizawai* 7.29 towards *S. littoralis* is due to the product of a specific gene, *cryIC*. As a consequence, the presence in strain *aizawai* 7.29 of other crystal proteins which are not specifically active against this insect species could decrease the toxicity of the native crystals of strain *aizawai* 7.29 towards this insect.

The amino-terminal domain of the CryIC protein, which corresponds to the active part of the molecules is significantly different (55%) from the other delta-endotoxins belonging to this family. The similarities (45%) are clustered in five conserved boxes that could be necessary to confer the molecule's biological activity. However, as the mechanism of action of these toxins is still unknown, such hypotheses are only tentative and one approach for testing the role of the various regions described above would be to construct a series of chimaeric genes in which one or more of the variable domains are modified or substituted and then to examine their properties.

Another approach would be to make site directed mutagenesis of these domains in order to

identify the sequences required for the toxicity or specificity. It must be noted that these studies will be facilitated by the genetic tools, vectors, and electoporation techniques (Lereclus et al., 1989), which are now available to reintroduce cloned genes in *B. thuringiensis.*

The *cryIC* crystal protein gene encodes a polypeptide that is much more active against larvae of *S. littoralis* and *M. brassicae* than are the other delta-endotoxins. This gene could be used for improved biological control of these insect pests via its manipulation:

This gene could be used to overproduce the toxin in *Bacillus subtilis* or other microorganisms in which expression vectors are available. Such microorganisms could be directly involved in producing insecticidal preparations for commercial use.

This gene could also be reintroduced in *B. thuringiensis* or related species such as *B. cereus*, by transformation and/or electroporation procedures. Such ways will allow to produce the toxin in large amounts without prior modification of the natural promoter region of the *B. thuringiensis* delta-endotoxin genes and generate derived strains having enhanced potencies against *S. littoralis* and *M. brassicae.*

Another interesting way would be to introduce and express the gene in plants susceptible to *S. littoralis* and *M. brassicae.*

Finally *in vitro* and *in vivo* recombination between this toxin gene and delta-endotoxin genes presenting different toxic specificities could lead to the construction of new genes encoding hybrid toxins with a wide activity spectrum active against selected target insects.

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