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MULTIPLE DELTA-ENDOTOXIN GENES IN *BACILLUS THURINGIENSIS* STRAINS ACTIVE AGAINST LEPIDOPTERAN SPECIES OF THE NOCTUIDAE FAMILY

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The insecticidal properties of *B. thuringiensis* species are mainly attributed to the parasporal crystal, also designated as δ-endotoxin that is synthetized and accumulated during the sporulation phase. Most of the known *B. thuringiensis* isolates display toxicity against larvae of more than 200 species of lepidoptera. Differences in susceptibility of lepidopteran larvae to the parasporal crystals from various isolates are one of the aspects of the high selectivity of the δ-endotoxin toward insect species (Dulmage et al., 1981). For a long time it is known that crystals are composed of basic protein subunits of MM 130-140 kDa which are protoxin molecules activated *in vitro* by gut proteases to give soluble products ranging from 60 to 70 kDa that are true toxin molecules. Such toxic units were identified with the N-terminal half of the protoxin molecule.

It is now well established that the δ-endotoxin genes are generally located on large plasmids with masses higher than 30 Mdal; in several cases a chromosomal localization of the gene was also reported (Klier et al., 1982). Furthermore, it has been demonstrated that in some strains, several copies of the crystal gene are present at different locations (Kronstad et al., 1983). Theses observations are consistent with the fact that this gene is frequently associated with the transposable elements Tn*4430* (Lereclus et al., 1984, 1986) and Is*231* (Mahillon et al., 1985) in the same host plasmids.

Owing to the high specificity of the δ-endotoxin, a number of lepidopteran species, among them insects belonging to the Noctuidae family, are poorly controlled with the commercial isolates of *B. thuringiensis* presently used as pesticides. Such is the case for the cotton leaf worm *Spodoptera littoralis*, a polyphagous insect which is a major pest of cotton and other crops of agronomical importance. A number of *B. thuringiensis* strains among serotypes H7 (subsp. *aizawai*) and H6 (subsp. *entomocidus*) were previously characterized for their significant level of activity against *S. littoralis* (Kalfon & De Barjac, 1985).

One of these strains, *B. thuringiensis* subsp. *aizawai* 7-29 was chosen as a tool for studying determinants involved in host specificity, particularly those that are responsible for the larvicidal activity against *S. littoralis*.

Components of the insecticidal crystals in B. thuringiensis *subsp.* aizawai *7-29* – In a previous study (Lecadet & Martouret, 1987) we have shown that crystals from *B. thuringiensis* subsp. *aizawai* 7-29 are specifically toxic against *S. littoralis*, although they display also significant activity toward other insect species such as *Pieris brassicae* L. These bipyramidal crystals are composed of at least two distinct protoxin components, seen as a doublet band corresponding to 130-135 kDa polypeptides after electrophoretic migration in SDS polyacrylamide gel. Under a variety of conditions believed to be similar to those prevailing *in vivo*, proteolysis of crystals led to multiple components with MM ranging from 60 to 70 kDa, designated as "K-60" fractions. Among components of the crude "K-60" fraction containing the active part of the protoxin molecules, we were able to separate and to characterize several distinct polypeptides, on the basis of their antigenic relationship and of their larvicidal properties (Lecadet et al., 1989).

A purified fraction specifically active against *P. brassicae,* consisted of a unique component of 61 kDa, homologous to the plasmid encoded crystal protein from *B. thuringiensis* subsp. *berliner* 1715 (Btb). A second purified fraction specifically active against *S. littoralis* was composed of 63 and 65 kDa polypeptides. Using a set of polyclonal antibodies it was demonstrated that these two components were not antigenically related each other and were different from the 61 kDa polypeptide. Two additional polypeptides of 68 and 70 kDa were generally associated with the 65 kDa component in a third purified fraction that was also active against *S. littoralis.* These two polypeptides appeared closely related to the 65 kDa component and they were thought to be intermediate proteolysed products.

From this study, we concluded that the activated "K-60" fractions derived from crystals of the *B. thuringiensis* subsp. *aizawai* 7-29, contained at least three types of δ-endotoxins originating from distinct protoxin molecules, one of them being responsible for the activity toward *S. littoralis.* These observations are in good agreement with results reported by other research groups (Jarrett et al., 1985; Knowles & Ellar, 1988; Höfte et al., 1988) which have suggested that crystals of the lepidopteran strains were composed of multiple δ-endotoxin molecules differing in their N-terminal active domain.

Cloning of multiple δ-endotoxin genes from B. thuringiensis *subsp.* aizawai *7-29* — DNA-DNA hybridization experiments, using the internal part (2 kb) of the *Btb* crystal protein gene as a probe, indicated that at least five δ-endotoxin genes were present in strain *aizawai* 7-29; one of which was borne by the 45 Md resident plasmid (pBT45). After it was transferred by mating into a *B. cereus* recipient strain, this plasmid induced the synthesis of crystalline inclusions that were specifically active against *P. brassicae* and poorly toxic against *S. littoralis.*

The crystal protein gene located on pBT45 was previously cloned in the recombinant plasmid pBT45-1 (Klier et al., 1985). As for the *berliner* strain, this plasmid crystal gene has been shown to be associated with the transposable elements Tn*4430* and Is*231* within a 18 kb *Bam*H1 DNA fragment whose restriction map is presented in Fig. 1. The toxin gene carried on plasmid pBT45 is structurally identical to the *berliner* plasmid gene characterized by Wabiko et al. (1986) and Höfte et al. (1986). A second gene contained in a 14 kb *Bam*H1 fragment isolated from total DNA of the *aizawai* 7-29 strain, has been cloned in the recombinant plasmid pHTA1. Again, we identified a crystal gene structurally identical to the *Btb* gene. The only differences (in size and restriction map) that have been observed between pBT45-1 and pHTA1 concerned the Tn*4430* flanking regions. The pHTA1 derived plasmid designated pHTA2 (Fig. 1) allowed a high level of expression of the δ-endotoxin gene in *E. coli,* leading to the synthesis of genuine crystals that were specifically active against *P. brassicae.* It appears therefore, that this type of crystal protein gene, a 5.3 class of gene as defined by Kronstad & Whiteley (1986) and its surrounding sequences are duplicated in strain *aizawai* 7-29.

Using defined parts of the pHTA2 DNA as a probe, a third gene contained in a 6 kb *Bgl*II fragment and a large part of a fourth gene contained in a 6.6 kb *Pst*I fragment were isolated, from total DNA of this strain, in recombinant plasmids pHTA4 and pHTA6 respectively. Structural analysis indicated that each of the two genes displays a unique physical map, different from those described for the lepidopteran, dipteran or coleopteran crystal genes already characterized. These two new genes, of presumed chromosomal origin, were shown to be located in close proximity (3 kb distant) and in the same orientation (Sanchis et al., 1988).

The gene cloned in the recombinant plasmid pHTA4 encodes a 130 kDa crystal protein that is not significantly toxic against *S. littoralis* or *P. brassicae.*

The fourth gene, we designated *Bta,* isolated in plasmid pHTA6 and in the derived plasmids pHT71 and pHT671, was obtained as a truncated crystal protein gene, encoding a 92 kDa protein that is specifically active toward *S. littoralis.* The gene product was also active against other species of the Noctuidae family *(Mamestra brassicae* and *Spodoptera exigua),* whereas it was not significantly toxic against *P. brassicae.* The determination of the nucleotide sequence of this gene indicated that the polypeptide contained a very high level of aminoacid substitutions in the N-terminal part of the protoxin molecule (Sanchis et al., 1989).

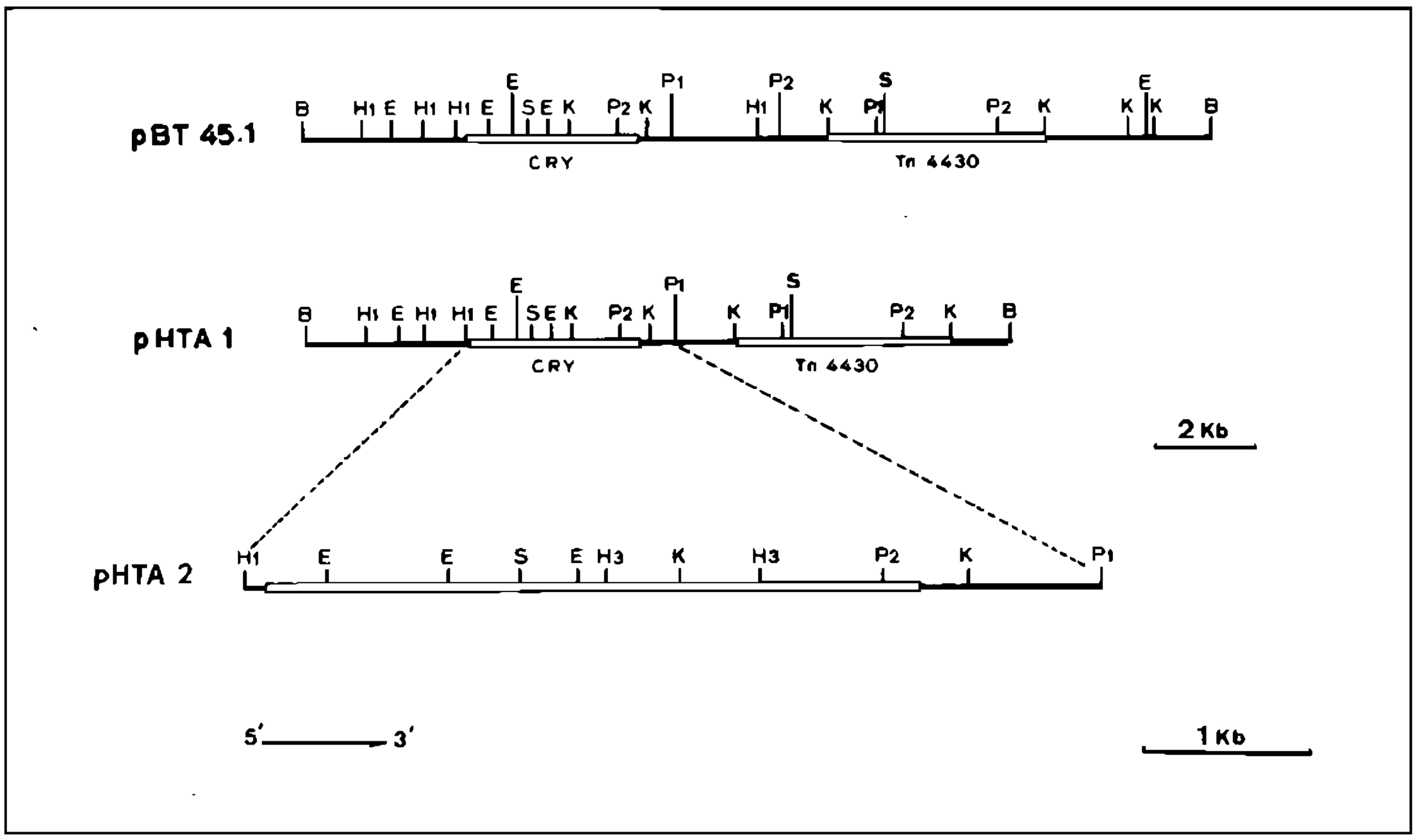

Fig. 1: physical map of the *Bam*H1 DNA fragments cloned respectively in plasmids pBT45-1, pHTA1 and the derived plasmids pHTA2. Abbreviations: B1: *Bam*H1; E: *Eco*R1; H1: *Hpa*I; H3: *Hin*dIII; K: *Kpn*I; P1: *Pst*I; P2: *Pvu*II; S: *Sst*I; cry: crystal gene.

As seen in Fig. 2, a pair of genes apparently homologous to those reported above, was also isolated from total DNA of *B. thuringiensis* subsp. *entomocidus* 601, a strain which also displays specificity toward *S. littoralis*.

Simultaneously, Visser et al. (1988) reported cloning of five crystal protein genes from DNA of the *B. thuringiensis* subsp. *entomocidus* 605; one of them was shown to code for a product with specificity toward *Spodoptera* species.

Identification of a δ-endotoxin gene product specific for S. littoralis – Four δ-endotoxin genes belonging to three different structural types have now been cloned from DNA of the *B. thuringiensis* subsp. *aizawai* 7-29, among them two new genes different of those already characterized. The expression products of these genes in *E. coli* were characterized and then compared with natural components of the insecticidal crystals from *B. thuringiensis* subsp. *aizawai* 7-29. The immunological detection of specific proteins and the quantitative estimation of their larvicidal activity in the protein extracts of the *E. coli* recombinant clones were the approaches which unabled us to differenciate the products of the cloned genes and to identify them with components of the "K-60" fractions. Results are summarized in the Table.

The crystal protein genes carried by recombinant plasmids pBT45-1 and pHTA2 encode 130 kDa proteins that display complete homology with the *Btb* gene product. These proteins were antigenically related with the 61 kDa polypeptide already characterized as a component of the "K-60" fractions derived from crystals of the *aizawai* strain. The target insect was *P. brassicae*.

The pHTA4 recombinant plasmid directs the synthesis of a 130 kDa protein that is different of the other gene products. We have argument for suggesting that this crystal protein is antigenically related to the 63 kDa component of the "K-60" fractions. The target insect has not yet been determined.

The 3' truncated δ-endotoxin gene designated as *Bta*, that is carried on the recombinant plasmids pHT71 and pHT671, determines the synthesis of a 92 kDa protein with high activity against *S. littoralis*. This polypeptide, in which the N-terminal active domain of the protoxin is entirely included, is not related to the other

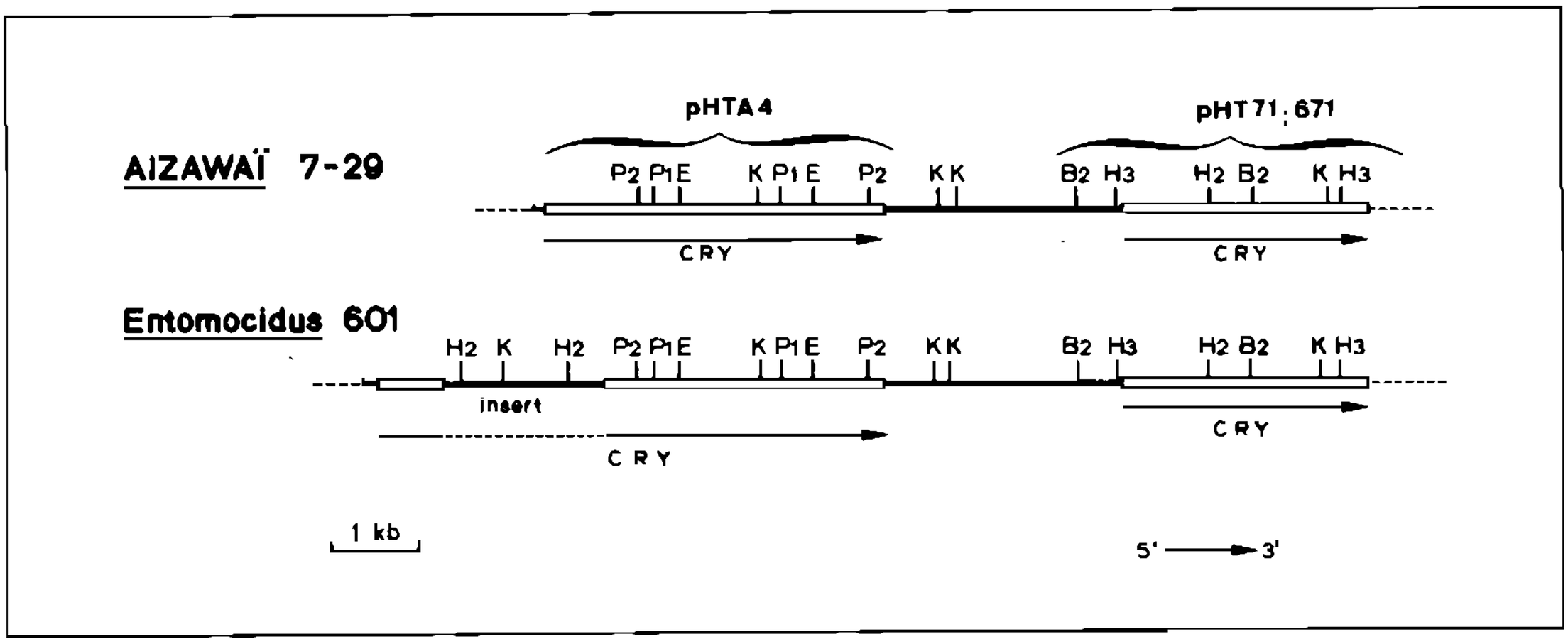

Fig. 2: physical map of the two new types of δ-endotoxin genes isolated from strains *aizawai* 7-29 and *entomocidus* 601. Abbreviations: B2: *Bgl*II; E: *Eco*R1; H3: *Hind*III; H2: *Hinc*II; K: *Kpn*I; P1: *Pst*1; P2: *Pvu*II.

TABLE

Characterization of the crystal protein genes from *B. thuringiensis aizawai* 7-29 and their products after cloning in *E. coli*

Recombinant plasmids	Origin of cloned DNA	Type of crystal protein gene	Encoded crystal protein in *E. coli*	Target insect	Homologous component among Bt "K-60" toxic units
pBT 45-1	plasmid	*Btb* (5,3 class of gene*)	130 kDa	*P. brassicae*	61 kDa
pHT A-2	presumed chromosomal	*Btb*	130 kDa	*P. brassicae*	61 kDa
pHT A-4	"	non identified	130 kDa	non identified	presumed 63 kDa
pHT 71	"	*Bta* (new class of gene)	92 kDa	*S. littoralis*	*65*, 68 and 70 kDa
pHT 671	"	*Bta*	92 kDa	*S. littoralis*	*65*, 68 and 70 kDa

* As defined by Kronstad & Whiteley (1986).

The two genes *Bta* and *Btb* mentioned above and in the text are now designated as *Cry*IC and *Cry*IA (b) respectively, according to the new classification proposed by Höfte & Whiteley (1989).

gene products, whereas homology was found with components of the *aizawai* crystals: more precisely, with a 135 kDa protoxin molecule and with the 65, 68 and 70 kDa components of the "K-60" fractions. Furthermore, the 92 kDa gene product can be proteolytically converted into a 65 kDa polypeptide through 68 and 70 kDa intermediates.

Measuring toxicities against two insect species, in terms of LC_{50} ratios, was a determinant tool used in the course of this study. Another decisive element for characterizing the gene products was the quantitative estimation of their toxic activity in terms of specific proteins. Based on immunoprecipitation experiments and owing to the fact that bioassays were performed by the forced feeding technique, (on fifth instar larvae) it was feasible to estimate the toxicity in terms of lethal doses (LD_{50} in μg per gram of larvae) and to compare the values with those of *B. thuringiensis* crystals and their derivatives. In this way it was demonstrated without any ambiguity that the *Bta* gene directs the synthesis of a δ-endotoxin that is very specific for *S. littoralis*, whereas the crystal protein genes of the *Btb* type determine products that are specific for *P. brassicae*.

Conclusion and Perspectives — In *B. thuringiensis* subsp. *aizawai* 7-29, multiple δ-endotoxin genes of different structural types direct the synthesis of several δ-endotoxins with different specificities, three of them were identified as components of the insecticidal crystals. The expression product of one of these genes was shown to be responsible for the larvicidal activity against *S. littoralis*.

As a consequence, the presence in strain *aizawai* 7-29 of other proteins that are not specifically toxic for *S. littoralis* could act in modifying the resulting activity of crystals. Whether the different components work independently or synergistically to determine the host specificity is a question that remains unsolved. To elucidate this point it would be necessary to test various mixtures of individual components.

Among other possible developments and applications involving the *Bta* gene, several lines of research can be drawn: overproducing the toxin in *E. coli* or other microorganisms, reintroducing the gene in *B. thuringiensis*, and modifying the gene in order to generate derived strains having enhanced potencies against *S. littoralis* (or Spodoptera species). Finally the *Bta* gene, or new derived genes, could be good candidates in view of constructing transgenics plants that are known to be severely damaged by this pest insect.

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