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CHARACTERIZATION OF GENES FROM *BACILLUS THURINGIENSIS ISRAELENSIS* ENCODING TOXINS WITH DIFFERENT SPECIFICITIES TOWARDS MOSQUITO LARVAE

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Biochemical analysis and cloning experiments by different groups indicate that 5. *thuringiensis var israelensis (Bti)* crystals contain at least four main proteins of 28 kd, 68 kd, 125 kd, and 135 kd. Results on the biocidal activity of each of these polypeptides are sometimes contradictory. Synergistic activity between several combinations of different proteins has been proposed. The genes coding for th*eBti* crystal proteins are located on a resident plasmid of 72 Md. From this plasmid we isolated the genes encoding the 28 kd protein, the 125 and the 135 kd proteins in *Escherichia coli* (Bourgoeuin et al., 1986, 1988; Delecluse et al,, 1988). Bioassays performed on mosquito larvae with the recombinant clones showed that:

— the 28 kd polypeptide is cytolytic.

— the 125 kd polypeptide is active against larvae of *Aedes aegypti, Anopheles stephensi* and *Culex pipiens.*

— the 135 kd polypeptide is active on larvae of *Ae. aegypti, An. stephensi* but not on larvae of C. *pipiens.*

To isolate *the Bti* crystal protein genes, DNA libraries of the 72 Md plasmid were established in *Escherichia coli.* Recombinant clones were screened by immunoassay with a serum directed against the solubilized crystals.

RESULTS

Characterization of the 28 kd protein gene

From an EcoRI library, recombinant plasmid pCB4 was isolated (Fig. 1). The E. *coli* recombinant clone synthesizes a 28 kd polypeptide at low level which is not active on either *Aedes aegypti* or *Culex pipiens* larvae. To enhance the expression level of the 28 kd protein gene, it was further cloned into the shuttle vector pHV33 and introduced into B. *subtilis.* In this host bacterium, cytolytic activity was detected but no larvicidal activity.

Characterization of the 135 kd protein gene

Plasmid pRX8 which contains the gene encoding 135 kd polypeptide was isolated from an *EcoRl* library (Fig. 1). The putative coding sequence was localized by subcloning experiments. Interestingly, the DNA fragment cloned in pRX8 contains sequences previously described by Thome *et. al* (1986) as ORF_1-ORF_2 adjacent to the 135 kd protein gene (see Fig. 1). These authors found that ORF_1-ORF_2 products were toxic to *Ae. aegypti* larvae.

Western blot analysis of cells containing either the 135 kd protein gene or both the 135 kd protein gene and ORF_1-ORF_2 showed that the major product expressed is a 135 kd protein. ORF_1-ORF_2

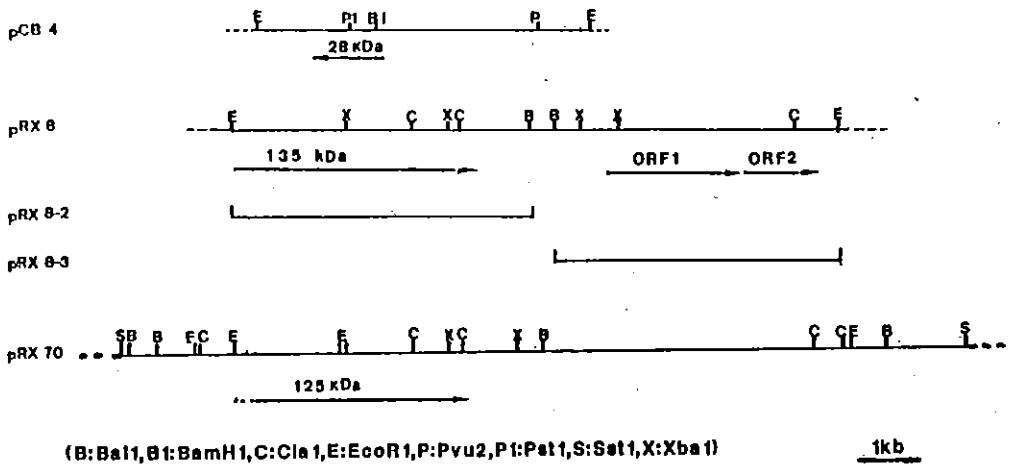

Figure 1.

products were poorly detected suggesting that these ORFs are expressed at low level, or that their products are not recognized by the antiserum used.

Bioassays performed on *Ae aegypti*, *C. pipiens* and *An. stephensi* larvae, with recombinant clones harbouring the 135 kd gene alone (RX8-2) or both (RX8), showed that clone RX8 is active against larvae of the three mosquito species, but that RX8-2 is only active against *Ae. aegypti* or *An. stephensi* larvae (Table 1). Clone RX8-3 displays no activity on *C. pipiens* and *An. stephensi*, and is weakly active on *Ae. aegypti* larvae. However when clone RX8-2, harbouring the 135 kd protein gene, was mixed with RX8-3, harbouring ORF_1-ORF_2 the toxicity to *C. pipiens* larvae was restored, suggesting that ORF_1-ORF_2 products act synergistically with the 135 kd protein on this mosquito species. Further subcloning experiments showed that only the ORF_1 product is involved in the synergism. On the other hand, comparison of amino acid sequences deduced from DNA sequences (Sekar and Carlton, 1986; Thorne et al., 1986; Ward and Ellar, 1987) suggest that ORF_1-ORF_2 is a modified 130 kd protein.

Delineation of the active moiety of the 135 kd protein was performed by successive deletions affecting either the 3' end or the 5' end of the gene. Bioassay experiments indicated that the toxic moiety of the protein is encoded by the 5' region of the gene. It corresponds to a polypeptide of approximately 65 kd delimited by lysine and arginine residues at position 50 and 627 respectively, in the amino acid sequence deduced from the DNA sequence published by Sekar and Carlton (1986).

Characterization of the 125 kd protein gene

An *Eco*RI fragment which contains a region homologous to the 3' region of the 135 kd protein gene was isolated during the cloning of the 135 kd gene. It encodes a *Bti* crystal related protein of 56 kd and could correspond to the 3' region of another 130 kd protein. From an *Sst*I plasmid library the entire gene was isolated in plasmid pRX70 (Fig. 1). Its 5' region is different from the 5' region of the 135 kd gene. This new gene encodes a 125 kd polypeptide detected by Western blot analysis.

The activity of the 125 kd protein was determined by bioassay on *Ae. aegypti*, *C. pipiens* and *An. stephensi* larvae. This polypeptide is toxic to the three mosquito species although the activity level

TABLE 1
Specificity of cloned toxin proteins

E. coli clone	Gene product	*Ae. aegypti*	*An. stephensi*	*C. pipiens*
CB4	28 kd	–	–	–
RX8	135 kd 58 kd	+++	+++	+++
RX8-2	135 kd	+++	+++	+/–
RX8-3	58 kd	+	–	–
RX8-2 + RX8-3	135 kd 58 kd	ND	ND	+++
RX70	125 kd	++	++	++

is lower than that obtained with the 135 kd protein (Table 1). This could be due to a difference in the expression level of the respective genes in *E. coli.*

DISCUSSION

Our results show that the *Bti* crystal proteins which we studied, have specific host range activity and suggest that they play different roles in the toxicity of *Bti* to mosquito larvae.

As the cloned 28 kd protein was shown to be hemolytic but not larvicidal to *Aedes* and *Culex*, its role in the *Bti* toxicity is probably a nonspecific cytotoxicity. Visser *et al.* (1986) also indicate that the purified 28 kd is not active against *Anopheles stephensi* larvae but is hemolytic. An overview of the published results shows that the 28 kd protein has rarely been found to be larvicidal at low concentration. Several groups reported that no toxicity against *Aedes* (Chung and Hammock, 1985; Hurley et al., 1987) occurred at 25 µg/ml of purified protein. Furthermore in vitro toxicity to cell lines from different origins including mammalian cells has been reported (Thomas and Ellar, 1983). Nevertheless, contradictory results were obtained either with cloned gene product (McLean and Whiteley, 1987; Ward et al., 1984) or with the purified protein (Thomas and Ellar, 1983). For this latter reference the 28 kd was in fact an enriched fraction and the activity detected is probably due to the synergistic activity occurring between the 28 kd and the 68 kd as described by Wu and Chang (1985).

Cloning experiments demonstrate that Bti possesses two different genes encoding high molecular weight proteins of 125 kd and 135 kd respectively which are both larvicidal but have different host range specificities. The 135 kd protein is active against *Ae. aegypti* and *An. stephensi* but not *C. pipiens* larvae, whereas the 125 kd is active against the three mosquito species. However, toxicity to *C. pipiens* larvae can be obtained due to a synergistic activity occurring between the 135 kd protein and ORF_1 product. The 135 kd and the 125 kd protein genes have been cloned by other teams and their products tested only against *Ae. aegypti* larvae (Angsuthanasombat et al., 1987; Sekar and Carlton, 1986; Ward and Ellar, 1988). The 125 kd and the 135 kd proteins are both present in the *Bti* crystals whereas the ORF1 product (a 58 kd protein) is detected only in very low amounts (Garduno et al., 1988). Nevertheless, the synergistic activity described with cloned products probably also occurs in *B. thuringiensis.*

Beside the proteins described above, *Bti* crystals also contain a 68 kd protein which accounts for

approximately 30% of the crystal protein. No hemolytic or nonspecific cytoxicity has been described. However, conflicting results on the larvicidal properties of this protein have been reported. Some authors found it highly active against *Ae. aegypti* larvae, whereas Wu and Chang (1985) proposed it acts synergistically with the 28 kd or the 130 kd polypeptides. Cloning experiments will resolve the issue of the intrinsic activity and specificity of the 68 kd protein.

In conclusion, cloning the different *Bti* crystal protein genes obviously provides important information on their product specificity and such information is necessary to improve *B. thuringiensis* as a biological control agent. Although data now exist for mosquito larvae, none are available for the specificity of the *Bti* toxins against black fly larvae. One way to improve the efficiency of *Bti* would be to overproduce the most active toxin with regards to the insect to be controlled. To achieve this aim transformation procedures are required for *Bti*. As there are now appropriate clones of all toxins, efficacious, suitable toxin overproducing strains will soon be available. Another useful approach would be to associate larvicidal toxins from *B. thuringiensis* and from *B. sphaericus* in the same organism. For that purpose *B. sphaericus* seems to be the organism of choice, as it does not release the toxic crystals into the medium and seems able to persist in treated pools. Transformation of *B. sphaericus* has been described and one can expect the development of recombinant strains. However, one limitation of the use of either *Bti* or *B. sphaericus* is that these organisms do not stay in the feeding area of the mosquito larvae. This disadvantage could be reduced by introducing the cyanobacterium genes encoding gas vesicle proteins or by introducing toxin genes into cyanobacteria able to grow in mosquito ponds.

Addendum: The gene encoding the 68 kd protein has been cloned (Donovan et al., 1988).

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