Cloning and Expression in *Escherichia coli* of an Insecticidal Crystal Protein Gene from *Bacillus thuringiensis* var. *aizawai* HD-133

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Using a gene probe derived from the cloned var. *sotto* insecticidal crystal protein (ICP) gene, we have cloned a *Bacillus thuringiensis* var. *aizawai* HD-133 ICP gene in *Escherichia coli*. The gene encodes a polypeptide that is toxic to Lepidoptera in vivo and in vitro. The protein is expressed at a level sufficient to produce phase-bright inclusions in recombinant *E. coli* strains, and these inclusions can be partially purified using discontinuous sucrose density gradients. Immunoblotting shows that the inclusions contain a 135 kDa polypeptide which reacts strongly with antiserum raised against the *B. thuringiensis* var. *kurstaki* HD-1 P1 polypeptide.

**INTRODUCTION**

Insecticidal crystal proteins (ICPs) are a group of bacterial toxins encoded by strains of *Bacillus thuringiensis* (BT) during sporulation (Bulla et al., 1980), and deposited in the cytoplasm as inclusions visible by phase-contrast microscopy. In commercial formulations these proteins are important for the control of agricultural pests (Burges, 1982). Different BT strains produce ICPs pathogenic to caterpillars (Lepidoptera) (Burges, 1982) mosquitoes and blackflies (Diptera) (Burges, 1982) and beetles (Coleoptera) (Krieg et al., 1983). Thus the studies of these ICP-encoding toxin genes are of considerable industrial interest.

Several ICP toxin genes have been cloned (Schnepf & Whiteley, 1981; Held et al., 1982; Kronstad & Whiteley, 1984; Klier et al., 1982, 1985; Shibano et al., 1985; Ward et al., 1984). The DNA sequences of the promoter and coding region of some ICP genes have been reported (Schnepf et al., 1985; Adang et al., 1985; McLinden et al., 1985; Shibano et al., 1985; Mahillon et al., 1985; Waalwijk et al., 1985; Ward & Ellar, 1986). The differences in length of HindIII restriction fragments containing the 5' ends of a number of ICP genes were used to group them into three classes termed the '4-5-, 5-3- and 6-6-kb class' (Kronstad & Whiteley, 1986). However, to date this classification of ICP genes applies only to var. *kurstaki* and var. *thuringiensis*.

Expression of ICP genes in BT begins at about stage II of sporulation (Bulla et al., 1980; Wong et al., 1983; Ward & Ellar, 1986) and the synthesis continues until late sporulation. Expression of ICP toxin genes has been observed during vegetative growth of *Bacillus subtilis* (Shivakumar et al., 1986; Ward et al., 1986). In the recombinant strain of *Escherichia coli*, the ICP gene of var. *kurstaki* HD-1-Dipel was expressed at all stages of growth (Wong et al., 1983).

BT strains from serotype 7 *aizawai* are of great interest for several reasons. Knowles et al. (1986) found that BT var. *aizawai* was active against a broad range of lepidopteran and dipteran cells *in vitro*, including cells from *Spodoptera frugiperda*. Recently, in a very thorough study, Lecadet & Martouret (1987) showed that among a range of BT isolates, an *aizawai* strain 7-29 (originating as HD-137 in the culture collection of Dr H.T. Dulmage, USDA, Brownsville, Texas, USA) was the most active against the cotton leaf worm, *Spodoptera littoralis*. This important pest is not adequately controlled by the existing commercial preparations of BT.

**Abbreviations**: BT, *Bacillus thuringiensis*; ICP, insecticidal crystalline protein.
Table 1. Recombinant plasmids containing inserts of BT var. aizawai HD-133 DNA

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Derivation</th>
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<tbody>
<tr>
<td>pCB1</td>
<td>14 kb BglII fragment which contains an entire ICP toxin gene cloned into the BamHI site of pUC18</td>
</tr>
<tr>
<td>pCB2</td>
<td>16 kb SsrI fragment which contains the N-terminus of the ICP toxin gene cloned into the SstI site of pUC18</td>
</tr>
<tr>
<td>pCB3</td>
<td>5.5 kb SsrI fragment which contains the C-terminus of the ICP toxin gene cloned into the SstI site of pUC18</td>
</tr>
<tr>
<td>pCB4</td>
<td>3.8 kb DraI fragment which contains the entire ICP gene, cloned into the SmaI site of pUC18</td>
</tr>
<tr>
<td>pCB5</td>
<td>pCB1 with the 6 kb BglII-PvuII fragment downstream of the ICP toxin gene deleted</td>
</tr>
<tr>
<td>pCB6</td>
<td>4 kb HinII fragment of pCB1 cloned into the SmaI site of pUC18</td>
</tr>
<tr>
<td>pCB7</td>
<td>pCB2 with the 11 kb SsrI-BamHI fragment upstream of the N-terminus deleted</td>
</tr>
<tr>
<td>pCB8</td>
<td>pCB3 with the 1.75 kb HindIII-SsrI fragment of the C-terminus deleted</td>
</tr>
<tr>
<td>pCB9</td>
<td>pCB4 with the 1.7 kb EcoRI-DraI fragment of the N-terminus deleted</td>
</tr>
<tr>
<td>pCB10</td>
<td>pCB4 with the 1.5 kb Clal-DraI fragment of the N-terminus deleted</td>
</tr>
<tr>
<td>pCB11</td>
<td>pCB4 with the 1.7 kb HindIII-DraI fragment of the C-terminus deleted</td>
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</table>

These data suggest that certain aizawai strains may synthesize a novel toxin with high activity against Spodoptera. The native crystal proteins isolated from var. aizawai HD-133 and several other aizawai strains contain more than one polypeptide of approximately 130 kDa (unpublished data). Whether these distinct crystal proteins contribute independently or synergistically to the activity spectrum remains to be determined. As part of an attempt to answer this question, we report here the cloning of an ICP gene from var. aizawai HD-133 and expression of the gene in E. coli both in vivo and in vitro. ICP inclusions were observed in recombinant E. coli strains. Characterization of the partially purified ICP inclusions from recombinant E. coli cells is reported.

METHODS

Bacterial strains and plasmids. Bacillus thuringiensis var. aizawai HD-133 and HD-229 were obtained from the culture collection centre, USDA, Brownsville, Texas, USA. ICP inclusions extracted from var. HD-229 were used as additional molecular mass markers in immunoblotting experiments. Escherichia coli TG1 = K12, Δ(lac-pro) supE thi hsdD5F' traD36 proA+ B+ lacY1 lacZAM15 (Gibson, 1984), was used as a cloning host. Plasmid pUC18 (Yanisch-Perron et al., 1985) was used as a cloning vector. The details of the construction of recombinant plasmids and derivatives are shown in Table 1.

Enzymes, hexanucleotide primers and radiolabelled compounds. Restriction enzymes were obtained from either New England Biolabs or Bethesda Research Laboratories. Klenow fragment of DNA polymerase and phage T4 DNA ligase were obtained from Dr T. Hunt, University of Cambridge, UK. The random hexanucleotide primers were from Pharmacia. Lysozyme was from BDH. [35S]Methionine and [α-32P]dATP were from Amersham.

Restriction enzyme digestions and ligations were carried out as described by Maniatis et al. (1982).

Extraction of plasmid DNA. Plasmid pUC18 was prepared from E. coli TG1 by an alkaline/SDS lysis method (Birnboim, 1983). Extraction and purification of plasmids from BT var. aizawai HD133 was based on the method described by Maniatis et al. (1982) for the extraction of large plasmids, with some modifications. A 4 ml volume of an overnight culture of var. aizawai HD-133 was inoculated into 40 ml CCY medium (Stewart et al., 1981) and grown to late-exponential phase at 30 °C. The cells were harvested and resuspended in 4 ml lysis buffer (10% w/v sucrose, 50 mM-Tris/HCl pH 8, 20 mM-EDTA) containing 1 mg lysozyme ml⁻¹ and incubated at 37 °C for 30 min. SDS was added to 4% (w/v) and the suspension was incubated at 37 °C for 30 min or until the cells were lysed. Then NaCl was added to a final concentration of 1 M and the solution placed on ice for at least 2 h. After centrifugation at 17000 g for 30 min, the supernatant was extracted twice with phenol/chloroform (1:1, v/v) followed by ethanol precipitation. Plasmid DNA was resuspended in TE (10 mM-Tris/HCl pH 8.0, 1 mM-EDTA) and stored at 4 °C.

Preparation of probe and in situ hybridization. The method of 'oligoprimer labelling' of DNA fragments was carried out as described by Feinberg & Vogelstein (1983). In situ hybridization of dried agarose gels was carried out by a modification (Earp et al., 1987) of the method of Tsao et al. (1983).

SDS-PAGE. This was done by the method of Thomas & Ellar (1983).
In vitro gene expression and immunoprecipitation. The coupled E. coli transcription–translation system was as described by Pratt (1984) and immunoprecipitation of products synthesized in vitro was as described by Ward et al. (1984), using var. kurstaki HD-1 antiserum (the generous gift of Dr B. H. Knowles) (Knowles et al., 1984), and var. israelensis 27K antiserum (the generous gift of Dr E. S. Ward) (Ward et al., 1984).

Cloning of the ICP gene. Total plasmid DNA isolated from var. aizawai HD-133 was restricted with BglII, DraI or SstI. The restricted DNA fragments were resolved by electrophoresis through 0.8% agarose. The gel was dried and hybridized in situ with ‘oligoprimer-labelled’ var. sotto ICP gene (2.8 kb) (Shibano et al., 1985). Restriction fragments which hybridized with the probe were purified by elution onto DEAE paper (Dretzen et al., 1981). Purified DNA fragments were then ligated into pUC18 and the resulting ligation mixtures used to transform E. coli TG1 to ampicillin resistance (100 µg ml⁻¹). Plasmid DNA extracted from pools of 10 recombinants was analysed by agarose gel electrophoresis followed by hybridization with ‘oligoprimer-labelled’ var. sotto probe. Pools which contained sequences homologous to this probe were subdivided into individual recombinants. Further hybridization analysis enabled recombinants harbouring the ICP gene to be isolated.

Immunoblotting. Protein samples were analysed by SDS-PAGE and immunoblotting after transfer to nitrocellulose filters (Schleicher & Schuell) by the method of Thomas & Ellar (1983). Horse-radish peroxidase conjugated anti-rabbit immunoglobulin (Sigma) was used to detect bound antibody (Hawkes et al., 1982).

Preparation of cell extracts from E. coli. The method used was adapted from that of Shivakumar et al. (1986). Recombinants were grown for 24 h in 100 ml 2 x TY medium containing 100 µg ampicillin ml⁻¹ (2 x TY medium contains 10 g tryptone, 10 g yeast extract and 5 g NaCl in 1 l distilled water); 10 ml of the culture was kept for immunoblotting and the remaining 90 ml was retained for in vitro toxicity tests. For immunoblotting the culture was centrifuged and the pellet washed twice in PESP buffer (50 mM-NaH₂PO₄ pH 7.0, 5 mM-EDTA, 80 mM-NaCl, 2 mM-phenylmethylsulphonyl fluoride). The cells were resuspended in 1 ml PESP buffer and frozen at −80 °C for 20 min. Then 1 ml 2 x gel sample buffer (4% w/v SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.125 M-Tris/HCl pH 6.8) was added, followed immediately by heating at 100 °C for 10 min. Insoluble material in the lysates was removed by centrifugation and 50 µl of the supernatant was loaded onto an SDS-PAGE gel. For in vivo toxicity assays the 90 ml sample of the culture was centrifuged and the pellet washed twice in sterile distilled water. Finally the cells were resuspended in 2 ml sterile distilled water and disrupted by sonication as described by Ward et al. (1984). The cell extract was then used in vivo toxicity tests.

Purification of ICP from E. coli. For in vitro toxicity assays, cultures (100 ml) were prepared and sonicated as described by Ward et al. (1984). The disrupted cell suspension was centrifuged and the pellet, containing approximately 500 µg of crystal protein inclusions, was resuspended in 5 ml sterile distilled water. The inclusions were separated on discontinuous sucrose gradients as described by Thomas & Ellar (1983), harvested, washed three times in distilled water, and resuspended in distilled water. Samples were stored at −20 °C.

In vivo toxicity assays. Cell extracts (100 µl) containing approximately 225 µg crystal protein inclusions ml⁻¹ were filtered on each side of 4.5 cm diameter cabbage leaf discs. The discs were dried in air and then placed in a ventilated Petri dish with 12 nd–3rd instar Pieris brassicae larvae. The number of dead larvae was recorded after 48 h incubation at room temperature.

In vitro toxicity assays. A 100 µg sample of purified ICP was solubilized by suspension in 100 µl freshly prepared 50 mM-Na₂CO₃/HCl pH 10.5 plus 10 mM-dithiothreitol for 2 h at 37 °C. The soluble fraction was activated by incubating it for 30 min at 37 °C with gut extract from P. brassicae (Knowles et al., 1984), at a protein :gut extract ratio of 10 : 1 (v/v). Toxicity of the activated toxin against Charistoneura fumiferana CF1 cells was determined as described by Knowles et al. (1986).

RESULTS AND DISCUSSION

Cloning and restriction mapping of the ICP gene from BT var. aizawai HD-133

The DNA probe from BT var. sotto encoding an ICP active against Lepidoptera (Shibano et al., 1985) hybridized with 14 kb BglII, 16 kb and 5.5 kb SstI and 3.8 kb DraI DNA fragments from BT var. aizawai HD-133 (data not shown). These DNA fragments were cloned into pUC18, yielding recombinant plasmids pCB1, pCB2, pCB3 and pCB4, respectively. The restriction maps of the inserts in all four recombinants that are given in Fig. 1 show that this ICP gene differs from the var. sotto ICP gene from which the probe was derived. The different sizes of the C-terminal HindIII fragments of the var. aizawai and var. sotto ICP genes (Fig. 1) indicate that the region of least homology is located in the C-terminal region of the toxin.

Characterization and gene expression of the ICP coding region in E. coli

Plasmids pCB1, pCB2, pCB3 and pCB4 were used to prime protein synthesis in vitro (Fig. 2). The products were immunoprecipitated with var. kurstaki HD-1 antiserum, but not with var.
israelensis 27K antiserum. The major immunoprecipitated products synthesized when plasmids pCB1, pCB3 and pCB4 were used in this system were approximately 90–100 kDa in size (Fig. 2). However, the major immunoprecipitated product of pCB2 was approximately 40–60 kDa (Fig. 2). These data suggested that recombinant pCB2 probably carries only a part of the ICP gene from var. aizawai HD-133, resulting in a truncated gene product. DNA extracted from all four recombinants, however, when used in the in vitro system, primed the synthesis of multiple low-molecular-mass polypeptides which were immunoprecipitated by the var. kurstaki HD-1 antiserum. Probably, this is due to premature termination of protein synthesis in the in vitro system. This has been observed for gene products with molecular masses greater than 70 kDa (Pratt, 1984).

Expression of the ICP gene was also analysed in vivo (Fig. 3) as described in Methods. In vivo, the cloned recombinants pCB1 and pCB4 produced a major polypeptide of 135 kDa that was immunoprecipitated by antiserum raised against the var. kurstaki HD1 N-terminal 65 kDa portion of the P1 lepidopteran-specific toxin (Knowles, 1984). In contrast, pCB2 produced a 55–60 kDa polypeptide (Fig. 3) which also cross-reacted with the var. kurstaki antiserum. These results, in conjunction with the hybridization data (Fig. 4), suggest that pCB2 encodes the N-terminal region of the ICP gene. The fact that no breakdown products of this smaller polypeptide were observed in immunoblotting after in vivo expression suggests that this 55–60 kDa polypeptide is relatively resistant to proteolysis, as has been found for the 65 kDa insecticidal N-terminal fragment from var. kurstaki HD-1 (Knowles et al., 1984). Recombinant pCB3, which hybridized with the C-terminal region of the var. sotto probe (Fig. 4), yielded immunoprecipitable products in vitro (Fig. 2) but not in vivo (Fig. 3). The reason for the in vitro expression is not known, although aberrant initiation and termination of protein synthesis has
Cloning of ICP gene from BT var. aizawai

Fig. 2. In vitro expression and immunoprecipitation of the ICP gene product of BT var. aizawai HD-133. Proteins produced from recombinant plasmids in the E. coli in vitro coupled transcription-translation system were reacted with antiserum raised against var. kurstaki HD-1 and var. israelensis 27 K ICPs. Total and immunoprecipitated gene products were separated by 13% (w/v) SDS-PAGE. Gels were fixed and treated in 1 M-sodium salicylate for 30 min. Dry gels were exposed to X-ray film for 24 h or longer at -80 °C. Tracks: 1, 2, 11 and 12, the total products of pCB4, pCB1, pCB2 and pCB3, respectively; 3, 5, 7 and 9, the products of pCB4, pCB1, pCB2 and pCB3, respectively, precipitated with var. kurstaki HD-1 antiserum; 4, 6, 8 and 10, the products of pCB4, pCB1, pCB2 and pCB3, respectively, precipitated with var. israelensis 27K antiserum. The major immunoprecipitable gene product of each recombinant plasmid is arrowed.

frequently been observed using the E. coli in vitro system (E. S. Ward, personal communication) and it is possible that a new initiation site which can only be recognized by the coupled transcription-translation system was generated during the insertion of the DNA fragment into the vector. Support for this hypothesis comes from the observation that removal of the HindIII fragment from pCB3, yielding pCB8 (Fig. 4), abolished gene expression completely (data not shown).

DNA isolated from recombinant pCB4 hybridized with both the N- and C-terminal regions of the var. sotto probe (Fig. 4). In addition, expression of pCB4 both in vitro and in vivo yielded a 135 kDa protein which reacted with var. kurstaki antiserum. This suggests that recombinant pCB4 harbours an intact ICP gene.

The level of expression from the recombinant plasmids in E. coli was not affected by addition of the lacZ inducer IPTG (data not shown). Recombinant plasmids pCB6, pCB2 and pCB7 were expressed as efficiently as the others in spite of the fact that the reading orientation (Fig. 4) of the inserts in these clones was in the opposite direction to that of the lacZ vector promoter. This implies that promoter sequences for the BT var. aizawai ICP gene are being recognized by the E. coli RNA polymerase. Wong et al. (1983) observed that BT var. kurstaki utilized two adjacent transcriptional start points during in vivo transcription of an ICP gene while a unique start point
Fig. 3. Immunoblotting of cell extracts from recombinant E. coli strains. Cell extracts from recombinant E. coli strains were analysed by 10% (w/v) SDS-PAGE and the proteins transferred to nitrocellulose membrane filters and immunoblotted with var. kurstaki HD-1 antiserum as described in Methods. Tracks: 1, var. aizawai HD-229 crystal; 2, var. kurstaki HD-1 crystal; 3, extracts from E. coli harbouring pSE2 (var. sotto clone); 4, 5, 6 and 7, extracts from E. coli harbouring pCB3, pCB2, pCB1 and pCB4 respectively; 8, molecular mass markers; 9, extracts from E. coli harbouring pUC18.

Fig. 4. Characteristics of recombinant plasmids pCB1-4 and their derivatives. The E. coli coupled transcription-translation system and immunoprecipitation of the gene products with var. kurstaki HD-1 antiserum were used to study ICP gene expression in vitro. Immunoblotting of cell extracts from E. coli harbouring different recombinant plasmids incubated with var. kurstaki HD-1 antiserum was used to study ICP gene expression in vivo. A 700 bp EcoRI fragment from the N-terminal part and a 780 bp EcoRI–ClaI fragment from the C-terminal part of the var. sotto ICP gene were used as probes to hybridize with restricted DNA fragments from the recombinant plasmids. Experimental details are given in Methods. The symbols + and − indicate positive and negative results of the assays respectively. Arrows above each recombinant plasmid indicate the direction of transcription from the vector promoter. NA, Not analysed.
Cloning of ICP gene from BT var. aizawai

Fig. 5. Hybridization pattern of recombinant plasmid pCB4 with var. sotto probe. A 2.8 kb Nsil-NruI intragenic fragment of the var. sotto ICP gene was used to hybridize with endonuclease-restricted pCB4 DNA: lane 2, SstI; 3, HindIII; 4, Clal; 5, SstI/HindIII; 6 SstI/Clal; 7, HindIII/Clal. Lane 1 contains unrestricted pCB4 and lane 8 contains a DNA restricted with HindIII. (a) Ethidium-bromide-stained 0.8% agarose gel; (b) in situ hybridization of an identical gel using the 2.8 kb ICP gene of var. sotto as probe. The 1.2 kb Clal fragment and the 1.0 kb HindIII fragment which hybridized with the probe are indicated.

was used in vivo by E. coli. This suggests that different sigma-factor subunits are being used to transcribe the same gene. Further work is necessary to investigate the nature of ICP gene transcription in both E. coli and Bacillus strains.

When a 2.8 kb Nsil-NruI intragenic fragment (see Fig. 1) of the var. sotto ICP gene (Shibano et al., 1985) was used to probe endonuclease-restricted pCB4 DNA (Fig. 5), only a 1.2 kb Clal fragment (N-terminus) and a 1.0 kb HindIII fragment (C-terminus) hybridized with the probe in HindIII-Clal-restricted pCB4 DNA. A 0.65 kb HindIII fragment (Fig. 5, lanes 3, 5 and 7) which extends from an intragenic HindIII site to a pUC18 polylinker HindIII site was located downstream of the 1.0 kb HindIII fragment, and did not react with the probe. This is probably
Fig. 6. Phase-contrast microscopy of phase-bright ICP inclusions. The growth conditions of cells containing plasmid pCB4 and the procedure for purification of ICP inclusions from *E. coli* harbouring the same recombinant plasmid are described in Methods. (a) Phase-bright ICP inclusions in *E. coli* containing recombinant pCB4; (b) partially purified ICP inclusions from *E. coli* containing recombinant pCB4.

due to the fact that this 0.65 kb *HindIII* fragment is beyond the region of the 2.8 kb intragenic *sotto* probe. A *ClaI-HindIII* fragment which is less than 0.56 kb size, located between the 1.2 kb *ClaI* fragment and the 1.0 kb *HindIII* fragment, probably electrophoreses off the end of the gel, and hence cannot be seen in Fig. 5. Whether this *ClaI-HindIII* fragment hybridizes with the probe remains to be determined.

The restriction map (Fig. 4) and the hybridization results indicate that the ICP gene of BT var. *aizawai* HD-133 may correspond to a 5.3 kb class gene (Kronstad & Whiteley, 1986), since a 5.1 kb *HindIII* fragment containing the N-terminal region of the ICP gene was found in recombinant pCB1. Furthermore, the 14 kb *BglII* fragment of the recombinant pCB1 is considerably smaller than the corresponding *BglII* fragment of this class of gene. This difference between the ICP gene of var. *aizawai* HD-133 and the 5.3 kb class gene of var. *kurstaki* may contribute to the different insecticidal activity of these two strains.

**Extraction of ICP from *E. coli* cells harbouring recombinant plasmids**

Under the growth conditions used, more than 95% of *E. coli* recombinants harbouring pCB4 produced ICP inclusions which were visible by phase-contrast microscopy within 24 h of growth (Fig. 6a). By contrast, only approximately 30% of recombinants harbouring pCB1 produced phase-bright inclusions, and these were considerably smaller than those in cells containing pCB4 (data not shown). Thorn *et al.* (1986) reported that insertion of transposon Tn5 between an upstream A+T-rich region and the promoter sequences, or deletion of these upstream A+T-rich sequences, enhanced the expression of a cloned BT var. *kurstaki* ICP gene in recombinant *E. coli* cells. From examination of the BT var. *sotto* ICP gene (Shibano *et al.*, 1985), the analogous upstream A+T-rich sequences have been deleted in the construction of pCB4 (Fig. 1) and therefore ICP gene expression in *E. coli* containing pCB4 may be enhanced for similar reasons. These data suggest that upstream sequences may function as negative regulatory elements in expression of these genes. The recombinant pCB2, which contains only the N-terminal portion of the gene (Fig. 4), also produced ICP inclusions (data not shown). However, these inclusions were much smaller than those in cells containing plasmids pCB1 and pCB4. No
Cloning of ICP gene from BT var. aizawai

Fig. 7. Immunoblotting of the activated solubilized ICP purified from recombinant E. coli strains. Immunoblotting of a 10% (w/v) SDS-PAGE gel incubated with var. kurstaki HD-1 antiserum. Methods for the solubilization and activation of the ICP are given in Methods. Tracks: 1, extracts from E. coli harbouring pUC18; 2, activated ICP inclusions from E. coli harbouring recombinant pCB1; 3, ICP inclusions from E. coli harbouring recombinant pCB1; 4, var. aizawai HD-229 crystal; 5, var. kurstaki HD-1 crystal; 6, activated ICP inclusions from E. coli harbouring recombinant pCB4; 7, ICP inclusions from E. coli harbouring recombinant pCB4.

Table 2. Toxicity tests with extracts prepared from strains of E. coli carrying various plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>In vivo assay (P. brassicae larvae)*</th>
<th>In vitro assay (% lysis of C. fumiferana cells)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCB1</td>
<td>12/12</td>
<td>25.6 ± 1.08</td>
</tr>
<tr>
<td>pCB2</td>
<td>0/12</td>
<td>No lysis</td>
</tr>
<tr>
<td>pCB3</td>
<td>0/12</td>
<td>No lysis</td>
</tr>
<tr>
<td>pCB4</td>
<td>12/12</td>
<td>31.3 ± 0.72</td>
</tr>
<tr>
<td>pUC18 (control)</td>
<td>0/12</td>
<td>No lysis</td>
</tr>
<tr>
<td>No plasmid†</td>
<td>0/12</td>
<td>No lysis</td>
</tr>
</tbody>
</table>

* Cell extracts (100 μl) containing approximately 225 μg crystal protein inclusions ml⁻¹ were brushed on each side of 4.5 cm cabbage leaf discs. The fraction represents the number of 2nd-3rd instar larvae killed after 48 h at room temperature. The experiments were repeated three times and gave the same results.

† Purified crystal protein inclusions were used for the in vitro assays. Approximately 50 μg soluble crystal protein inclusion activated with P. brassicae gut extracts was added to 1 ml CF1 cells (1 x 10⁶ cells ml⁻¹). The percentage of lysis was assessed by inability to exclude Trypan Blue in 60 min incubation. No lysis indicates that no cytopathic effect was observed within 3 h.

‡ An appropriate volume of buffer, gut extract and cell extract without plasmid was used as an additional control.

ICP inclusions could be observed in cells containing pCB3. Attempts were made to extract the crystal protein inclusions from recombinant E. coli strains by using discontinuous sucrose gradients (Thomas & Ellar, 1983). Crystal protein partially purified from E. coli (Fig. 6b) was solubilized (see Methods) and the solubilized crystal protein proteotoxin activated by treatment with P. brassicae gut extract (Knowles et al., 1984). This proteolytic degradation yielded a polypeptide of 60 kDa which showed strong immunological reaction with var. kurstaki HD-1 P1 antiserum (Fig. 7).
Toxicity of ICPs extracted from E. coli

The results of toxicity tests of the ICP toxin in vivo and in vitro are shown in Table 2. Cell extracts from E. coli were used for the in vivo assays, with cell extracts from E. coli harbouring only pUC18 as the control. Extracts from recombinants pCB1 and pCB4 killed P. brassicae within 48 h, whilst pCB2, pCB3 and control extracts had no effect (Table 2). Recombinant pCB2 was non-toxic in spite of the fact that a 55-60 kDa polypeptide was produced (Fig. 3). This implies that a region in the C-terminal half of the gene may be essential for the toxicity. Similar results have been observed by Schnepf & Whiteley (1985). Partially purified crystal protein inclusions were used for in vitro toxicity tests. The results obtained using C. fumiferana cells are shown in Table 2: pCB1 and pCB4 toxin lysed about 30% of the cells within 60 min but no further lysis was observed after prolonged incubation.

We are grateful to Dr E. S. Ward and Dr B. H. Knowles for valuable discussions during the course of this work.

REFERENCES


Ward, E. S. & Ellar, D. J. (1986). Bacillus thuringiensis var. israelensis δ-endotoxin nucleotide sequence and characterization of the transcripts in Bacillus thuringiensis and Escherichia coli. Journal of Molecular Biology 191, 1–11.


