# Cloning and expression in *Escherichia coli* of the insecticidal δ-endotoxin gene of *Bacillus thuringiensis* var. *israelensis*

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Recombinant plasmids containing the mosquitocidal δ-endotoxin gene were constructed by inserting *Hind*III fragments of the *Bacillus thuringiensis* var. *israelensis* 72–75 Md plasmid in to the *Escherichia coli* vector pUC12. Two recombinants producing the 26000 Da δ-endotoxin (pIP173 and pIP174) were identified by screening clones in an *E. coli* in vitro transcription-translation system. Both recombinants were 12.4 kb chimaeric plasmids comprising pUC12 and a common 9.7 kb *Hind*III fragment of the *B. thuringiensis* plasmid. The 26000 Da polypeptide synthesis in vivo from pIP174 transformed into *E. coli* JM101 was lethal to mosquito larvae and cytotoxic to mosquito cells in vitro. The biological authenticity of the cloned product was further confirmed by demonstrating that the cytotoxicity of the polypeptide was neutralised by antiserum to the authentic δ-endotoxin or by preincubation with excess toxin receptor. Transcription of the recombinant δ-endotoxin gene in *E. coli* appears to utilise a *Bacillus* promotor sequence(s) rather than the pUC12 β-galactosidase promotor.

Bacillus thuringiensis *var.* israelensis      *δ-Endotoxin gene*      *Gene cloning*      *Insecticide*      *Plasmid*      *Mosquito*

## 1. INTRODUCTION

The gram-positive bacterium *Bacillus thuringiensis* var. *israelensis* [1] produces a cytolytic protein δ-endotoxin that is lethal to the larvae of mosquitoes and blackfly (*diptera*) [2,3]. This δ-endotoxin is synthesised during sporulation as a parasporal crystalline protein inclusion [4,5]. A combination of the potent activity of this *israelensis* δ-endotoxin against *dipteran* disease vectors, with the fact that protein δ-endotoxins produced by other serotypes of *B. thuringiensis* are highly toxic to a wide range of *Lepidopteran* pest insects [6], has provoked considerable scientific and commercial interest in these bacteria over the past 30 years.

Recent reports of the isolation and expression of the δ-endotoxin gene of *B. thuringiensis* var. *kurstaki* [7,8] and var. *berliner* [9] indicate that the

δ-endotoxin gene may be variously located on plasmid DNA, chromosomal DNA, or both [7–11]. In the case of *B. thuringiensis* var. *israelensis*, two independent studies of strains cured of one or more plasmids concluded that δ-endotoxin synthesis is critically dependent on the presence of a 72–75 Md (~110 kb) plasmid [12,13]. Using the newly discovered capacity of *B. thuringiensis* for plasmid transfer by a conjugation-like mechanism, authors in [11] have shown that one or more plasmids in several *B. thuringiensis* strains code for the δ-endotoxin structural gene. However, to date, transfer of plasmids from *B. thuringiensis* var. *israelensis* to other *B. thuringiensis* serotypes has not been achieved [11] and it therefore remains possible that the 72–75 Md plasmid encodes a regulator of δ-endotoxin production, rather than the structural gene.

We describe the resolution of this question by the isolation from the purified 72–75 Md plasmid of a 9.7 kb restriction fragment that encodes the *israelensis* δ-endotoxin gene.

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## 2. MATERIALS AND METHODS

### 2.1. *Bacterial strains and plasmids*

The strains used were: a derivative of *B. thuringiensis* var. *israelensis* IPS78 (Dr H. de Barjac, Institute Pasteur, Paris) that had been cured of several plasmids present in the wild-type strain [12]; *Escherichia coli* JM101 and plasmid pUC12 have been described previously [14].

### 2.2. *Isolation of plasmid DNA*

pUC12 plasmid was prepared from *E. coli* JM101 by a lysozyme-detergent lysis method [18] with the following modifications (J. Karn, personal communication): (i) Triton X-100 was used to a final concentration of 1% instead of sodium dodecyl sulphate (SDS); (ii) NaCl was omitted after Triton addition. Total plasmid DNA from *B. thuringiensis* var. *israelensis* was prepared by the method of [15]. The 72–75 Md plasmid was subsequently purified from total plasmid DNA by preparative vertical electrophoresis on 3 mm-thick 0.5% low gelling temperature agarose slabs (Seaplaque, FMC Colloids). Gel bands were visualised and excised as previously described [16,17]. Plasmid DNA from excised bands was purified by phenol/chloroform extraction and ethanol precipitation. Total CCC DNA for use in the in vitro transcription-translation system was purified on caesium chloride gradients as in [18].

### 2.3. *Cloning of DNA*

Vector (pUC12) and *B. thuringiensis* var. *israelensis* DNA (72–75 Md plasmid) was digested to completion with *Hind*III (New England Biolabs). The medium salt buffer of [18] was used for *Hind*III digestions, except that for DNA extracted from low gelling temperature agarose, bovine serum albumin (Sigma) was added to a final concentration of $100 \mu g \cdot ml^{-1}$. 25 ng restricted vector and 100 ng restricted 72–75 Md plasmid were ligated with T4 DNA ligase (New England Biolabs) at 15°C for 16 h, using the manufacturer's instructions for buffer, except that bovine serum albumin was used at a final concentration of $100 \mu g \cdot ml^{-1}$ [17]. Portions of the ligation mix were used to transform *E. coli* JM101 [19] and transformants were selected on L agar [18] containing $170 \mu g \cdot ml^{-1}$ ampicillin.

### 2.4. *Preparation of antibodies*

The protein $\delta$-endotoxin (26000 Da) was isolated from purified crystals of *B. thuringiensis* var. *israelensis* by preparative gel electrophoresis (to be described elsewhere). Purified $\delta$-endotoxin was mixed with Freunds complete adjuvant and antibodies raised by subcutaneous injection of this material into New Zealand White rabbits. Specificity of antisera was confirmed by Western blotting [20] using horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Sigma) to detect bound antibody [21].

### 2.5. *Analysis of recombinants*

Recombinant clones were analysed using an in vitro transcription-translation system [22]. DNA from individual recombinants or recombinant groups was extracted using a small-scale lysozyme–Triton plasmid preparation method ([18] and J. Karn, personal communication) and up to $5 \mu g$ DNA was added to the *E. coli* system. Products from the in vitro system were analysed using 13% acrylamide gels [23] and fluorography [24].

### 2.6. *In vitro toxicity assays*

Recombinants were grown for 16 h in L. Broth [18] containing $100 \mu g \cdot ml^{-1}$ ampicillin and harvested by centrifugation. Pellets from 1 l cultures were resuspended in 12 ml 50 mM $Na_2CO_3 \cdot HCl$, pH 10.5, and disrupted by sonication. Five periods of 30 s sonication with a 0.5-inch sonic probe (Dawe Instruments, London) operating at maximum intensity were sufficient to break more than 90% of the cells. The resulting lysate was incubated at 37°C for 1 h, and then saturated ammonium sulphate was added to a final concentration of 30%. The precipitate was pelleted by centrifugation and resuspended with 5 ml 50 mM $Na_2CO_3 \cdot HCl$, pH 10.5. 50–100 $\mu l$ of this suspension was added to a 4 cm Petri dish containing *Aedes albopictus* cells as previously described [2]. Results were recorded using phase contrast microscopy and an Olympus OM2 camera attachment.

### 2.7. *In vivo toxicity assays*

Recombinants were assayed for in vivo toxicity using a modification of the method of [25]. Recombinants were grown for 16 h at 37°C in L.

Broth [18] containing $100\ \mu g \cdot ml^{-1}$ ampicillin and harvested by centrifugation. Pellets from 100 ml culture were resuspended in 6 ml distilled water. 1.5 ml of this suspension was added to the cup containing 25 *A. aegypti* larvae [*A. aegypti* eggs were kindly supplied by Mr D. Funnell (Shell Research Ltd.)].

## 3. RESULTS AND DISCUSSION

Preliminary screening with restriction enzymes showed that when *Hind*III digested *B. thuringiensis* var. *israelensis* total plasmid DNA was added to the *E. coli* in vitro transcription-translation system, a single novel polypeptide identical in molecular weight to the authentic *israelensis* toxin was precipitated by antiserum raised to the purified 26000 Da $\delta$-endotoxin (fig.1). *Hind*III was therefore used to digest the purified 72–75 Md plasmid and the products ligated into *Hind*III digested pUC12 as described in section 2. A library of 450 colonies was selected after transformation of *E. coli* JM101 with the ligation mixture. Random analysis of 60 colonies from this library showed that 77% (46) were recombinants (not shown).

Plasmid DNA was then extracted from three groups of 150 clones and 5 $\mu$g DNA from each group added to the in vitro transcription-translation system. All three pools contained recombinant(s) that synthesised a 26000 Da polypeptide precipitable by the $\delta$-endotoxin antibody. The group containing the most 26000 Da protein detectable by immunoprecipitation was further subdivided using the *E. coli* in vitro system. DNA was isolated from groups of 25, then 5 and finally single recombinant clones and screened for toxin synthesis. In this way two toxin-coding recombinants pIP173 and pIP174 were identified from one of the groups of five clones (fig.1) and used for subsequent analysis.

Fig.2 shows the results of horizontal agarose gel electrophoresis of the covalent closed circular forms of pIP174 (lane a) and pUC12 (lane b) extracted from individual recombinants together with their *Hind*III digestion products. Digestion of pIP174 yielded a 9.7 kb insert (lane c) in addition to the 2.7 kb fragment derived from pUC12 (lane d). Identical results were obtained with *Hind*III digested pIP173 (not shown).
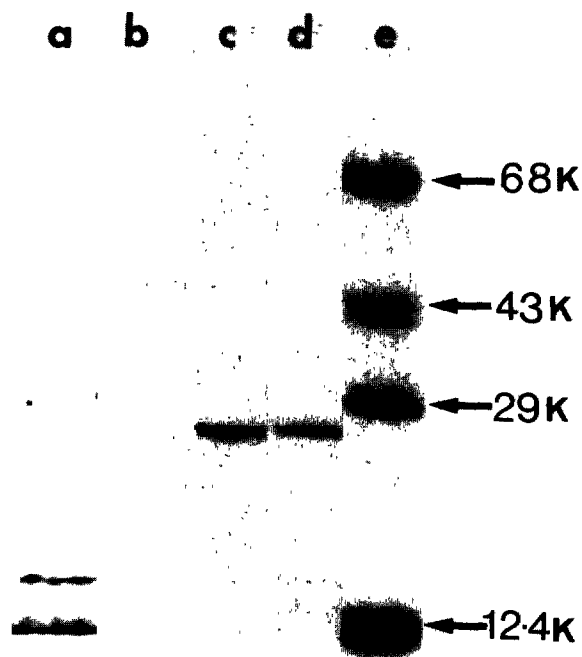


Fig.1. Fluorographs of SDS 13% polyacrylamide gels of the $^{35}$S-labelled polypeptides synthesised in the *E. coli* transcription-translation system primed with either *Hind*III digested total *B. thuringiensis* var. *israelensis* plasmid DNA, or plasmid pIP174 DNA, and supplemented with L-[$^{35}$S]methionine: Lane a, total products from pIP174 DNA; lane b, material precipitated from a by addition of preimmune serum; lane c, material precipitated from a by addition of antibody raised against the 26000 Da authentic *israelensis* $\delta$-endotoxin; lane d, material precipitated by antibody against the 26000 Da authentic *israelensis* $\delta$-endotoxin from the *E. coli* transcription-translation system primed with *Hind*III digested total *B. thuringiensis* var. *israelensis* plasmid DNA; lane e, molecular weight standards labelled with ethyl-[1-$^{14}$C]acetimidate hydrochloride (the generous gift of Dr J. Gray [22]).

Although the immunoprecipitation of the in vitro synthesized cloned product showed clearly that a 26000 Da polypeptide antigenically related to the *israelensis* $\delta$-endotoxin was encoded by the 9.7 kb insert, additional experiments were needed to confirm that the polypeptide was biologically active. Lysates were therefore prepared from 1 l cultures of colony 174 and control *E. coli* JM101 colonies containing pUC12 lacking any insert and assayed for toxicity in vitro as described in section 2.
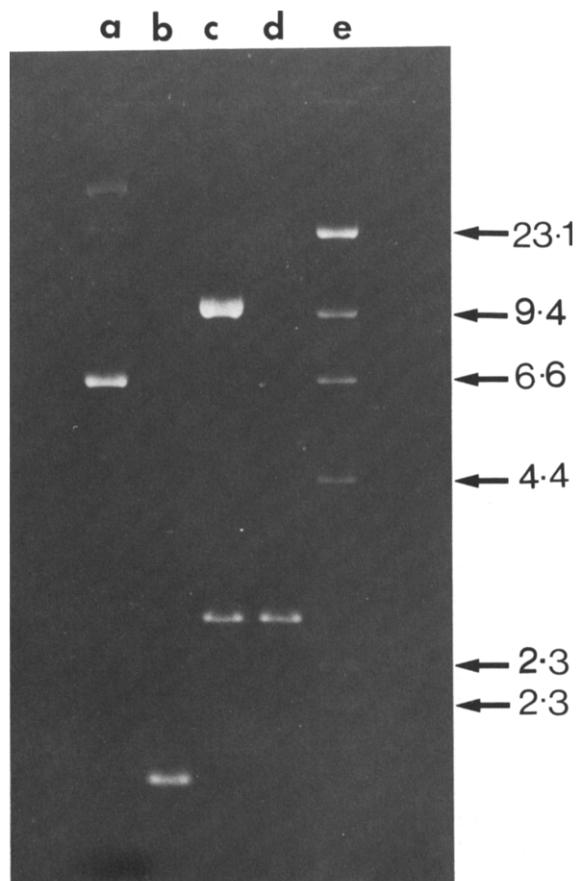
Fig.2. Agarose gel electrophoresis of covalent closed circular forms of pIP174, pUC12 and their HindIII digestion products: lane a, pIP174 (covalent closed circular form); lane b, pUC12 (covalent closed circular form); lane c, HindIII digested pIP174; lane d, HindIII digested pUC12; lane e, HindIII digested λDNA with fragment sizes on the right margin (in kb).

Protein extracted from colony 174 caused cytolysis of *Aedes albopictus* cells (fig.3) indistinguishable from that previously described for authentic δ-endotoxin [26]. An amount of this protein extract equivalent to 6 ml of the original *E. coli* culture produced typical cytotoxicity (cell detachment, rounding, swelling and granulation) after 4 h exposure to $5 \times 10^5$ *Aedes* cells, with complete lysis after 16 h. By comparison with dose response curves for authentic δ-endotoxin, this result indicates that the *E. coli* extract contains approximately 20 μg per ml of protein toxin. *Aedes* cells exposed to an equivalent protein extract from *E. coli* containing the vector pUC12 alone were unaffected even after prolonged exposure (24 h).

The authenticity of the pIP174 encoded polypeptide was further confirmed by demonstrating that the toxicity of the 174 lysate could be neutralised either by antiserum directed
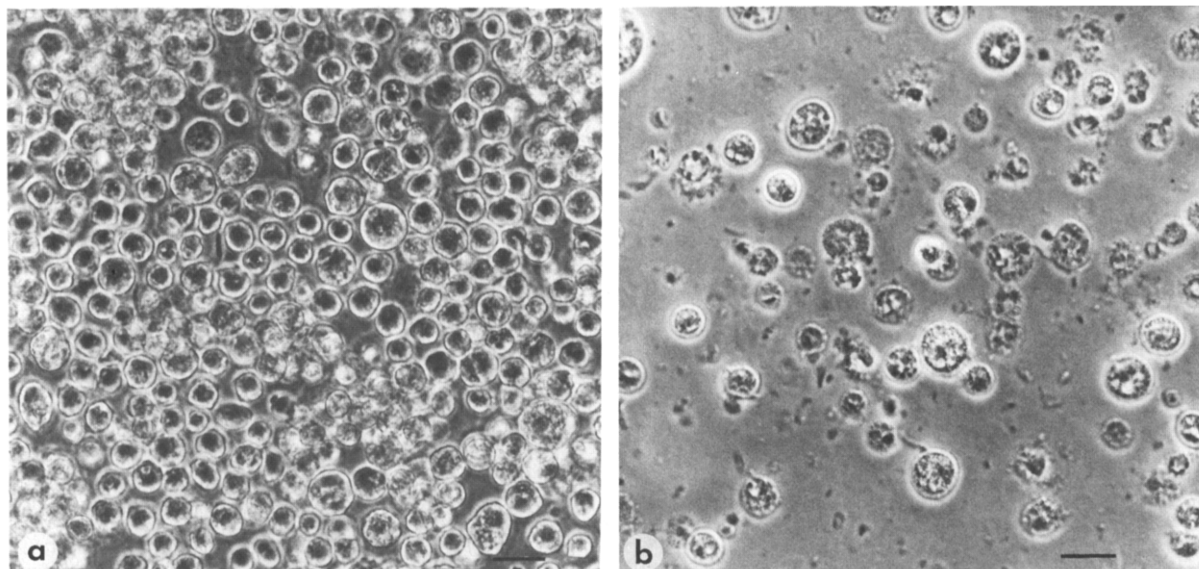


Fig.3. Phase contrast micrographs of *A. albopictus* cells: a, 24 h after addition of a lysate of *E. coli* JM101 containing pUC12 with no insert; b, 4 h after addition of a lysate of *E. coli* JM101 containing pIP174. Bar in each case represents 25 μM. (For experimental details see section 2.)

against the 26000 Da native δ-endotoxin, or by preincubation of the lysate with sonicated preparations of those phospholipids previously shown to be the cell membrane receptors for authentic δ-endotoxin [26]. As expected from the former result, addition of the δ-endotoxin-specific antiserum to the 174 lysate precipitated a 26000 Da polypeptide that was not detectable in control lysates (not shown).

Final confirmation of the biological authenticity of the cloned product was obtained from in vivo bioassays. 25 s instar *Aedes aegypti* larvae were killed in 4 h when fed an amount of *E. coli* containing pIP174 equivalent to 25 ml of original culture. In control experiments larvae fed equivalent amounts of *E. coli* JM101 containing pUC12 with no insert were unaffected.

Addition of a β-galactosidase inducer isopropyl-β-D-thiogalacto-pyranoside (IPTG) to cultures of *E. coli* JM101 containing pIP174 did not result in an increase in toxin production measured in vitro. This suggests that expression of the δ-endotoxin gene in *E. coli* is under the control of *B. thuringiensis* promoter sequences rather than the vector β-galactosidase promoter. In *B. thuringiensis* var. *israelensis* the 26000 Da polypeptide component of the crystalline inclusion represents approximately 15% of the total cell protein at the end of sporulation. As others [7–9] have observed for cloned lepidopteran-specific δ-endotoxin genes, the level of expression of the *israelensis* gene in *E. coli* is very low. This is not surprising in view of the lack of homology between *E. coli* consensus promoter sequences and promoters controlling *Bacillus* sporulation specific genes, exemplified here by the δ-endotoxin gene [27,28]. Experiments are now in progress to identify these transcriptional start sequences in the cloned *israelensis* δ-endotoxin genes.

## ACKNOWLEDGEMENTS

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