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Single Amino Acid Changes in the *Bacillus thuringiensis* var. *israelensis* δ-Endotoxin Affect the Toxicity and Expression of the Protein

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Site-directed mutagenesis has been used to change individual amino acids of the larvicidal 27,000 M_r δ-endotoxin of *Bacillus thuringiensis* var. *israelensis*. Basic and acidic residues have been systematically replaced by alanine, and the resulting mutant polypeptides analysed for cytolytic and larvicidal activity, and binding to phosphatidyl choline liposomes. Replacement of residues at positions 154, 163, 164, 213 and 225 results in proteins which accumulate as inclusions in recombinant *Bacillus subtilis* cells similar to the wild-type, but have considerably reduced *in-vitro* and *in-vivo* toxicity. One mutant (Glu45 to Ala45) results in a protein that has reduced activity *in vitro*, but retains wild-type larvicidal toxicity. In addition, seven other mutations of charged residues result in proteins which form small or no inclusions in recombinant cells, despite being produced at levels similar to the wild-type in six out of seven cases. In most instances, the toxicity of these aberrantly expressed proteins is considerably less than the wild-type, although one (Lys124 to Ala124) results in a polypeptide with approximately threefold increased activity *in vitro*. A secondary structural model is proposed to explain these observations.

1. Introduction

Strains of the spore-forming bacterium *Bacillus thuringiensis* synthesize cytoplasmic crystalline protein inclusions which are toxic to insect larvae (Luthy, 1980). The inclusion produced by *B. thuringiensis* var. *israelensis*, which kills mosquito and blackfly larvae (Goldberg & Margalitt, 1977; de Barjac, 1978), is irregular in shape and contains several polypeptides (Thomas & Ellar, 1983*a*) that have been shown to be larvicidal by a combination of biochemical and genetic approaches (Davidson & Yamamoto, 1984; Thomas, 1984; Armstrong *et al.*, 1985; Cheung & Hammock, 1985; Hurley *et al.*, 1985; Lee *et al.*, 1985; Sriram *et al.*, 1985; Wu & Chang, 1985; Visser *et al.*, 1986; Ward *et al.*, 1984; Waalwijk *et al.*, 1985; Bourgouin *et al.*, 1986; McLean & Whiteley, 1987). The gene encoding the 27,000 M_r major polypeptide in the *israelensis* inclusion has been cloned into *B. subtilis* where the protein accumulated as phase bright inclusions similar in appearance to, but smaller than, the var. *israelensis* crystal (Ward *et al.*, 1986). These inclusions, consisting solely of the 27,000 M_r protein, were shown to be both larvicidal and cytolytic (Ward *et al.*, 1986).

The nucleotide sequence of the 27,000 M_r δ-endotoxin has been reported (Waalwijk *et al.*, 1985; Ward & Ellar, 1986), and the hydropathy plot shows this protein to be highly hydrophobic, consistent with its postulated mode of action (Thomas & Ellar, 1983*b*). The protein has been shown to interact with specific plasma membrane phospholipids (Thomas & Ellar, 1983*b*) and it has been suggested that this polypeptide shares a common cytolytic mechanism with *B. thuringiensis* δ-endotoxins from other serotypes (Knowles & Ellar, 1987). It is proposed that these δ-endotoxins bind to receptors on the membrane and then interact to create a membrane hole or pore. The generation of these pores leads to colloid-osmotic lysis (Knowles & Ellar, 1987).

In an attempt to understand more of the

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interaction of the var. *israelensis* 27,000 M_r δ-endotoxin with target membranes, we have used *in-vitro* techniques to direct specific codon alterations in the cloned δ-endotoxin gene. Acidic and basic residues of this protein have been systematically changed to alanine, to investigate the role of charged residues in the interaction with polar phospholipid headgroups. To date, this is the first report in which mutagenesis of single amino acids has been employed to investigate structure–function relationships of a *B. thuringiensis* δ-endotoxin.

2. Materials and Methods

(a) *Strains and plasmids*

Strains of *Escherichia coli* used were: *E. coli* TG1=K12, Δ(*lac-pro*), *sup*E, *thi*, *hsd*D5/F'*tra*D36, *pro*A+B+, *lac*I^q, *lac*ZΔM15 (a generous gift from Dr T. Gibson; Gibson, 1984); and *E. coli* BMH 71·18 *mut*L (a kind gift from Dr G. Winter, MRC Laboratory of Molecular Biology, Cambridge). *B. subtilis* 168 Sueoka *trp*C2 and *B. subtilis* MB24 *met*C3, *rif*, *trp*C2 (generous gifts from Dr T. Leighton and Dr P. Piggot, respectively) were used as cloning hosts for preparation of the wild-type 27,000 M_r δ-endotoxin and mutant derivatives. Plasmid Camtaq 11 has been described (Ward *et al.*, 1986). Plasmid Cambtaq 6 is similar to Camtaq 11 except that the 1·2 kb *Taq*I fragment harbouring the δ-endotoxin gene is in the opposite orientation. Phages M13tg130 and M13tg131 (Amersham International) were used during the construction and sequence analysis of the mutants. pUC12 and pUC19 have been described (Messing, 1983; Yanisch-Perron *et al.*, 1985). The 17 base-pair oligonucleotides were synthesized by the phosphotriester method (Itakura *et al.*, 1975) using a Biosearch DNA synthesizer.

(b) *Enzymes and radiolabel*

Restriction enzymes (Bethesda Research Laboratories) and calf intestinal alkaline phosphatase (Boehringer-Mannheim) were used in the medium salt buffer described by Maniatis *et al.* (1982). Phage T4 DNA ligase and polynucleotide kinase were from New England Biolabs, and the Klenow fragment of DNA polymerase was prepared by Dr R. T. Hunt, University of Cambridge. [α-^{32}P]ATP (3000 Ci/mol) and [α-^{35}S]dATP were obtained from Amersham International.

(c) *Site-directed mutagenesis*

A 1·2 kb† *Taq*I fragment (Ward *et al.*, 1986) harbouring the 27,000 M_r δ-endotoxin gene was cloned in pUC12 (Messing, 1983) in both orientations. The 725 bp or 490 bp *Pst*I fragments containing a portion of the δ-endotoxin gene and either 5' or 3'-flanking regions, respectively, were generated using an intragenic *Pst*I site and the *Pst*I site in the polylinker of pUC12 (Messing, 1983). These 2 fragments were purified (Dretzen *et al.*, 1981) and ligated into the *Pst*I site of M13tg130 in both orientations, and recombinants producing single-stranded DNA containing the non-coding δ-endotoxin strand were used as a source of template to generate mutants. The oligonucleotides used in this study (only those which resulted in the production of proteins of altered activity/expression are shown), together with the amino acid changes which they directed were:

5' GTAATAACCGCTGCTGT 3'	(Arg25 to Ala25);
5' TCCTCAACAGCTAATGT 3'	(Arg30 to Ala30);
5' TTATCAATTGCGTAAT 3'	(Glu45 to Ala45);
5' ATACTAAAGGCTAGGGC 3'	(Arg78 to Ala78);
5' ATACAGTTGCTAACACT 3'	(Lys124 to Ala124);
5' CTTCATTTGCTTGAGTA 3'	(Lys154 to Ala154);
5' CAGTTTCCGCGCCCCAG 3'	(Lys163 to Ala163);
5' TAGCAGTTGCCTTGCCC 3'	(Glu164 to Ala164);
5' CTTGTTCCGCTACTGCT 3'	(Lys203 to Ala203);
5' AAAACTTGTGCCTTTAC 3'	(Glu204 to Ala204);
5' TCGCAGATGCTTGAATT 3'	(Asp213 to Ala213);
5' GTGCAAATGCCAAAGAT 3'	(Lys225 to Ala225);
5' TAGTAAGAGCTGCAATT 3'	(Asp240 to Ala240).

Mutagenesis experiments were carried out as described (Carter *et al.*, 1985), with the exception that the mutagenic primer alone was used to prime *in-vitro* DNA synthesis. The single-stranded DNA isolated from mutant plaques was used for dideoxy sequencing analysis (Sanger *et al.*, 1977; Bankier & Barrell, 1983), and corresponding replicative form (RF) DNA was used to construct a complete δ-endotoxin gene containing the required codon change in the chimeric plasmid Camtaq 11 or Cambtaq 6.

(d) *Recloning of mutagenized δ-endotoxin genes for expression in* B. subtilis

RF DNA harbouring the mutations described above was restricted with *Pst*I and 725 bp or 490 bp fragments gel-purified (Dretzen *et al.*, 1981) by electrophoresing the DNA on to Schleicher and Schuell NA45 paper. The fragments were then ligated into gel-purified *Pst*I 5·9 kb or *Pst*I 6·14 kb fragments derived from Camtaq 11 or Cambtaq 6, respectively, and consisting of pUC12 (Messing, 1983), pC194 (Horinouchi & Weisblum, 1982) and either a 480 bp or a 715 bp *Pst*I–*Taq*I fragment of var *israelensis* DNA. Chimeric plasmid DNA, initially constructed using *E. coli* as a cloning host, was analysed by restriction enzyme digestion, and the constructs harbouring complete mutant δ-endotoxin genes used to transform *B. subtilis* protoplasts (Chang & Cohen, 1979).

(e) *Purification of δ-endotoxin inclusions from recombinant* B. subtilis *cells*

Recombinant *B. subtilis* cells containing mutant δ-endotoxin genes ligated into chimeric plasmids were grown in 2×SG (Leighton & Doi, 1971) containing 4 μg chloramphenicol/ml. An overnight culture was diluted 1:10 into fresh media, and grown to an optical density of 0·6 at 600 nm. This exponentially growing culture was diluted 10-fold into fresh media and the growth of the cells monitored until spores and inclusions were released. Mutant proteins which accumulated as inclusions were isolated from spore/crystal mixtures using sucrose density gradient centrifugation (Thomas & Ellar, 1983*a*). Protein concentrations were determined by the method of Lowry *et al.* (1951).

(f) *Analysis of δ-endotoxin expression in recombinant cells*

Recombinant *B. subtilis* cells were grown as described above, and were harvested by centrifugation at various stages of sporulation, resuspended in solution I (Birnboim & Doly, 1979) containing 2 mg lysozyme/ml for 10 min at 20 °C, and then lysed by sonication as

† Abbreviations used: kb, 10^3 bases or base-pairs; bp, base-pair(s).

described (Ward *et al.*, 1984). Overnight cultures of recombinant *E. coli* cells in 2×TY (Bankier & Barrell, 1983) containing 100 μg ampicillin/ml were diluted 50-fold into fresh media, grown to an optical density of 0·8 to 1·0 at 600 nm, and the *lac*Z inducer isopropyl-1-thio-β-D-galactoside (IPTG) added to a final concentration of 0·5 mm. Cultures were grown for an additional 40 h before harvesting by centrifugation. Cells were lysed using the same method as for *B. subtilis*, with the exception that the incubation in solution I was carried out at 0 °C.

Cell lysates were analysed by polyacrylamide gel electrophoresis (Laemmli, 1970) and immunoblotting (Towbin *et al.*, 1979; Hawkes *et al.*, 1982).

(g) *Toxicity assays* in vivo

Purified δ-endotoxin inclusions, or lysates of recombinant *B. subtilis* cells, were assayed for toxicity *in vivo* using *Aedes aegypti* larvae (3rd instar) as described (Ward *et al.*, 1984). (*A. aegypti* eggs were kindly provided by Mr D. Funnel, Shell Research Ltd.)

(h) *Toxicity assays* in vitro

Inclusions purified from recombinants were assayed for toxicity *in vitro* using *A. aegypti* cells, *Anopheles gambiae* cells, *Culex quinquefasciatus* cells and human or rabbit erythrocytes as described by Thomas & Ellar (1983*a*). Protein was solubilized in 50 mM-Na_2CO_3HCl (pH 10·5) in the presence of 0·1 vol. of *A. aegypti* gut extract (generously supplied by Dr J. Horsnell). Insect cell lines were kindly provided by Drs B. Knowles, J. Horsnell, Mr T. Sawyer and Mr J. Carroll, and were grown as described (Knowles *et al.*, 1986).

(i) *Phospholipid binding assays*

Liposomes were prepared as described (Thomas & Ellar, 1983*b*) at 20 °C using phosphatidyl choline (type III S)/cholesterol/dicetyl phosphate in molar proportions of 3·5:0·5:1 (by vol.) at 20 °C. Incubations of solubilized δ-endotoxins with liposomes, and detection of toxin neutralization were carried out as described by Thomas & Ellar (1983*b*). Lipids and phospholipids were obtained from Sigma.

3. Results

(a) *Generation and analysis of the mutations*

Figure 1 shows the nucleotide sequence of the non-coding strand of the var. *israelensis* δ-endotoxin gene and 5′-flanking region, together with the amino acid sequence of the native protein. The locations of each of the *in-vitro* mutations are indicated. For each *in-vitro* mutation, the nucleotide sequence of a substantial portion of the structural gene adjacent to the directed mutations was determined, to ensure that secondary undesired mutations had not been generated during the oligonucleotide-directed mutagenesis procedure. Expression of wild-type and mutant δ-endotoxins in *B. subtilis* recombinants was analysed using

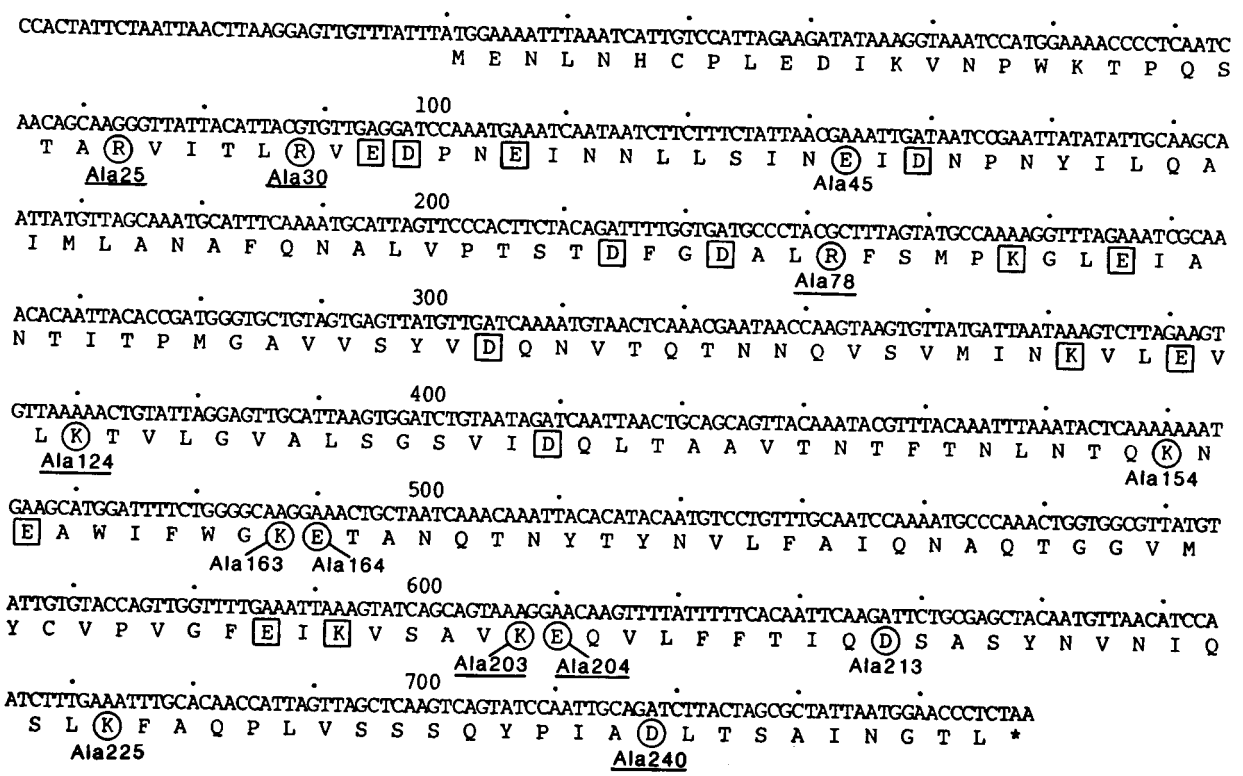

Figure 1. Nucleotide sequence of the var. *israelensis* 27,000 M_r δ-endotoxin gene, indicating the location of the codon changes directed by synthetic oligonucleotides (Materials and Methods). Mutations that affect the toxicity of the protein are indicated by circled amino acid residues with the designations shown; those that affect both toxicity and expression by circled amino acids and underlining; those that have no effect on the resulting protein by boxed residues.

Table 1
In-vitro *and* in-vivo *activities of mutant δ-endotoxins*

Mutant protein	Original Amino acid	*In-vitro* activity† *An. g.*	*A.a*	*C.q.*	R.b.c. (%)§	*In-vivo* activity‡ *A. aegypti*	PC binding
Ala45	Glu	26	31	56	12	125	No
Ala154	Lys	76	70	>100	0	>1000	No
Ala163	Lys	15	22	45	0	500	No
Ala164	Glu	19	21	>50	50	500	No
Ala213	Asp	45	40	>100	100	1000	No
Ala225	Lys	>100	>100	>100	0	>1000	No
Wild-type		4·2	4·9	7·5	100	125	Yes

An. g., Anopheles gambiae; A.a., Aedes aegypti; C.q., Culex quinquefasciatus.
PC binding, binding to phosphatidyl choline liposomes.
† LC_{50} values (in μg/ml) determined by uptake of Trypan blue after 40 min of assay (Knowles *et al.*, 1986).
‡ LC_{50} values (in ng/ml) after 24 h of assay.
§ Haemolytic activity as % of wild-type activity; 0% indicates that no haemolysis could be detected when the mutant polypeptide was used at a concentration of 60 μg/ml. (Haemolysis could be detected for the wild-type protein at a concentration of 7·5 μg/ml under the conditions of assay.)

phase contrast microscopy and immunoblotting of lysates of sporulating cultures (data not shown).

Of the mutated genes 21 out of 28 encoded δ-endotoxin which accumulated as phase bright inclusions identical in size and appearance to those described for the wild-type gene during late sporulation (Ward *et al.*, 1986). When these 21 types of mutant inclusions were purified and assayed, 15 (boxed amino acids in Fig. 1) showed identical toxicity *in vivo* and *in vitro* to the wild-type protein. The toxicity of the remaining six mutant proteins in this group was quite different from the wild-type (Table 1). For several of the charged residues shown to be important for toxicity in Table 1, the acidic (Glu45) or basic (Lys154, Lys163) residues were replaced by Asp (for Glu45) or Arg (for Lys154 and Lys163) through further rounds of site-directed mutagenesis. The resultant mutant proteins were indistinguishable from the wild-type polypeptide in all assays carried out. In addition to an analysis of their *in-vivo* toxicity and *in-vitro* cytolytic activity, the ability of each polypeptide to bind to unsaturated phosphatidyl choline liposomes was examined (Table 1), since it had been shown that this *in-vitro* binding was a reliable model of toxin–receptor interaction (Thomas & Ellar, 1983*b*).

A second group comprising 7/28 of the mutated δ-endotoxin genes (underlined in Fig. 1), encoded polypeptides which produced small or no visible inclusions in late sporulating cultures of recombinant *B. subtilis* (Table 2). For each of these mutants the level of δ-endotoxin expressed during early (stage III) and late stages (stage VI) of sporulation was analysed by immunoblotting. These stages of sporulation were selected because expression of the wild-type δ-endotoxin is readily detectable during stage III of sporulation and continues to be expressed during subsequent stages to reach a maximum level of accumulation at stage VI of sporulation (Ward *et al.*, 1986). With the exception of Ala78, which produced less 27,000 M_r protein (data not shown), the *B. subtilis* recombinants harbouring these mutant genes produce similar levels of δ-endotoxin to the wild-type (Table 2). Figure 2 illustrates this for mutants Ala124 and Ala204. In addition, for several of these mutant polypeptides, multiple polypeptides of molecular weight lower than 27,000 can be visualized on immunoblots, particularly for lysates of stage VI cells, suggesting that these mutant proteins are more susceptible to proteolysis than the wild-type (Fig. 2, compare lanes 4 and 6).

For the majority of these altered polypeptides which did not produce inclusions similar to those of the wild-type δ-endotoxin, it was not possible to

Table 2
Expression of mutant δ-endotoxin genes in recombinant B. subtilis *cells*

Mutant protein	Original amino acid	Level of expression† (%) Stage III	Stage VI	Phase bright inclusions
Ala25	Arg	5	10	None visible
Ala30	Arg	5	10	None visible
Ala78	Arg	5	2	Small inclusions
Ala124	Lys	5	10	Small inclusions
Ala203	Lys	5	10	Small inclusions
Ala204	Glu	5	10	Small inclusions
Ala240	Asp	5	10	Small inclusions
Wild-type		5	10	Large inclusions

† Expressed as estimated % of dry weight of bacterial cultures.

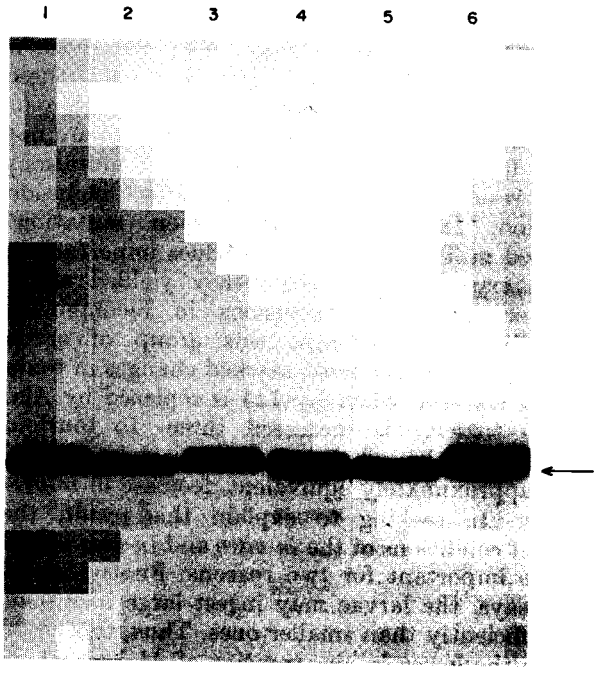

Figure 2. Analysis of expression of wild-type and mutant δ-endotoxins in recombinant *B. subtilis* cells using SDS/13% polyacrylamide gel electrophoresis followed by immunoblotting with antibodies raised against purified var. *israelensis* 27,000 M_r protein. Immunoblots are of lysates made from: lanes 1 to 3, stage III cells (200 μg of lysate loaded); and lanes 4 to 6, stage VI cells (100 μg of lysate loaded). Cells harbour the following (mutant) δ-endotoxins: lanes 1 and 4, Ala204; lanes 2 and 5, Ala124; lanes 3 and 6, wild-type. An arrow indicates the position of the 27,000 M_r δ-endotoxin.

obtain *in-vitro* and *in-vivo* toxicity data. For two mutants (Ala124 and Ala204), however, small inclusions could be purified, and their toxicity properties and binding to phosphatidyl choline are summarized in Table 3. For the remaining five polypeptides in this group, qualitative *in-vivo* assays indicate that Ala25 and Ala30 have toxicities similar to the wild-type, whereas the toxicities of Ala78, Ala203 and Ala240 are considerably less (data not shown).

Although the *in-vivo* activity of Ala45 is unchanged, the *in-vitro* activity is considerably reduced compared to the wild-type (Table 1). This allowed us to investigate further an earlier observation (Ward, 1986) that induction of a high level of wild-type 27,000 M_r δ-endotoxin expression had a deleterious effect on the growth of *E. coli*. It was suggested that this may be the result of membrane damage caused by interaction of the toxin with phosphatidyl ethanolamine in the *E. coli* membrane. We therefore examined the effect of cloning and expressing Ala45 in *E. coli*. An *Sst*I–*Hae*III fragment carrying the mutant δ-endotoxin gene was isolated, using a polylinker *Sst*I site and an *Hae*III site 35 nucleotides upstream from the toxin gene initiation AUG, and ligated into *Sst*I–*Sma*I restricted pUC19. *E. coli* cells harbouring the required *lacZ*-transcriptional fusion were identical in their growth rate, before and after induction, compared to cells harbouring pUC19. In addition, immunoblotting of lysates indicated than these cells synthesize a considerably higher level of mutant δ-endotoxin that cells harbouring the wild-type gene (Fig. 3).

4. Discussion

The results obtained from an analysis of the novel polypeptides are summarized in Tables 1 to 3. The data indicate that removal of glutamic acid at residue 45 causes an eightfold reduction in haemolytic activity without affecting the *in-vivo* activity. The *in-vitro* activity of this mutant against three mosquito cell lines is also considerably reduced. To substantiate the requirement for an acidic amino acid at this position, Glu45 was changed to Asp45. The resulting protein had the same properties as those of the wild-type δ-endotoxin (data not shown). Two possible reasons for the properties of Ala45 are: firstly, that an aberrant conformation resulting from the lack of charge at this position inhibits phosphatidyl choline binding *in vitro*, but removal of this region, perhaps by more extensive proteolysis in the insect gut, restores a conformation compatible with membrane binding and insertion; secondly, membrane components, in addition to phosphatidyl choline in the gut epithelium, could be important for *in-vivo*

Table 3
In-vitro *and* in-vivo *toxicities of mutants Ala124 and Ala204*

Mutant	Original amino acid	*In-vitro* activity† *An. g.*	*A.a.*	*C.q.*	R.b.c.§ (%)	*In-vivo* activity‡ *A. aegypti*	PC binding
Ala124	Lys	1·6	1·8	1·9	400	400	Yes
Ala204	Glu	14	36	>100	25	>1000	No
Wild-type		4·4	4·7	7	100	125	Yes

An. g., *Anopheles gambiae*; *A. a.*, *Aedes aegypti*; *C.q.*, *Culex quinquefasciatus*.
PC binding, binding to phosphatidyl choline liposomes.
† LC_{50} values (in μg/ml) determined by uptake of Trypan blue after 40 min of assay (Knowles *et al.*, 1986).
‡ LC_{50} values (in ng/ml) after 24 h of assay.
§ Haemolytic activity as % of wild-type protein activity.

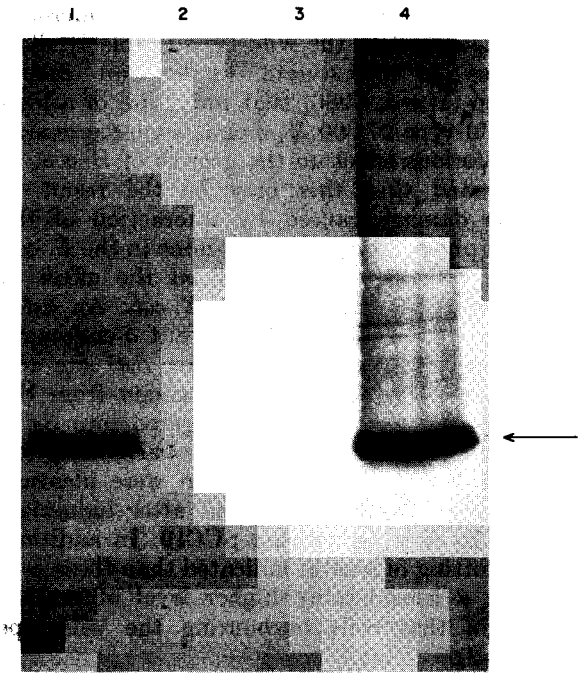

Figure 3. Expression of the 27,000 M_r δ-endotoxin and a mutant derivative in recombinant *E. coli* cells using SDS/13% polyacrylamide gel electrophoresis followed by immunoblots with antibodies raised against purified var. *israelensis* 27,000 M_r protein. Immunoblots are of lysates of cells harbouring *lacZ* transcriptional fusions to either the Ala45 mutant gene (lane 1), or the wild-type gene (lane 2), and cells harbouring pUC19 (lane 3). Cell lysate (200 μg) was loaded on to each lane; 12 μg of purified var *israelensis* 27,000 M_r protein were loaded on to lane 4. An arrow indicates the position of the 27,000 M_r δ-endotoxin.

activity, and the capacity of the protein to interact with these additional receptors may not have been affected in this mutant.

The higher level of expression of the Ala45 protein compared to the wild-type polypeptide in recombinant *E. coli* cells is of interest in two respects. Firstly, the phospholipid receptors of the 27,000 M_r protein (Thomas & Ellar, 1983*b*) are known to be abundant in the *E. coli* membrane (Birdsall *et al.*, 1975), and thus this amino acid change appears to affect the interaction. In support of this, toxin neutralization assays indicate that this mutant polypeptide does not bind to phosphatidyl choline liposomes (Table 1). Secondly, this *lacZ* fusion will provide a useful means of preparing mosquitocidal toxin from recombinant *E. coli* cells.

Five mutations involving replacement of acidic and basic amino acids (Ala154, Ala163, Ala164, Ala213 and Ala225) result in δ-endotoxins which show considerably reduced activity both *in vitro* and *in vivo*, suggesting that these residues are at positions in the tertiary structure which are critical for toxicity. This is supported by the observation (Table 1) that none of the mutant δ-endotoxins has retained the ability of the wild-type protein to bind to phosphatidyl choline, a target of the native toxin (Thomas & Ellar, 1983*a*). The fact that these proteins still form inclusions in recombinant *B. subtilis* cells suggests that these residues are not critical for this assembly. Similarly 15 mutations (boxed residues in Fig. 1) did not affect inclusion formation. In contrast, the seven mutations described in Table 2 may be residues important in protein crystallization, since they yielded either small or no visible inclusions in recombinant *B. subtilis* cells. Amongst this group of seven mutant proteins, the most marked changes in toxin activity are seen when Lys124 is replaced by Ala. *In-vitro* toxicity is increased three- to fourfold depending on the cell type, but this is accompanied by an approximately equivalent decrease in *in-vivo* toxicity. In seeking to explain this result, the different conditions of the *in-vitro* and *in-vivo* assays may be important for two reasons. Firstly, in *in-vivo* assays, the larvae may ingest large inclusions more efficiently than smaller ones. Thus, this could explain the lower *in-vivo* toxicity of Ala124, which assembles into relatively small inclusions. Secondly, conformational changes caused by mutations that confer an increased ability to bind to and lyse target cells *in vitro* may expose certain cryptic sites for gut proteases, resulting in cleavage to an inactive form *in vivo*.

Mutant Ala78 can be detected only at low levels in lysates of late sporulating *B. subtilis* cells, and yet assembles into small phase bright inclusions. This indicates that the high level of synthesis seen in mutants Ala124, Ala203, Ala204 and Ala240 is not a prerequisite for aggregation. This contrasts with other recombinant proteins (Mongkolsuk *et al.*, 1983) and is further evidence that specific regions of the var. *israelensis* δ-endotoxin are involved in the assembly of inclusions. The reasons for the low level of expression of the Ala78 protein are as yet unclear. The low level of expression of Ala78 becomes particularly noticeable during the later stages of sporulation when the proteases in *B. subtilis* are known to increase (Eaton & Ellar, 1974), suggesting post-translational instability.

Of interest with respect to inclusion formation is the observation that replacement of arginine residues at positions 25 and 30 does not affect the level of expression of the protein, but recombinant cells do not produce inclusions. As two 25,000 M_r proteins, derived from the 27,000 M_r δ-endotoxin by proteolytic cleavage at residues 29/30 and 31/32 have both mosquitocidal and cytolytic activity (Armstrong *et al.*, 1985), this suggests that Arg25 and Arg30 are involved in assembly of the δ-endotoxin inclusion. Moreover, mutation of these two residues does not appear to affect the *in-vivo* toxicity, which is consistent with the observations of Armstrong *et al.* (1985).

In an attempt to rationalize the data presented above, we have used a computer algorithm based on the method of Garnier *et al.* (1978) and other analytical methods (Taylor, 1987) to predict the

secondary structure of the 27,000 M_r δ-endotoxin (Fig. 4). Preliminary structural data using crystallographic studies have been reported by McPherson *et al.* (1987), and suggest that the protein contains a parallel or anti-parallel helix bundle as a major structural unit. The predicted model in Figure 4 is seen to contain two parallel helix bundles. The location of the mutations shown in Figure 1 are indicated on the proposed model. Data obtained from an analysis of the mutant proteins indicate that charged residue mutations that affect the activity are distributed throughout the polypeptide. The mutated residues analysed in Table 1, which have an effect on *in-vitro* and in 5/6 cases *in-vivo* activity, can be broadly divided into two classes: (1) those in coil/turn loops of the protein (Ala45, Ala213 and Ala225); and (2) those in the helix 3 region (Ala154, Ala163 and Ala164). Moreover, the charged residues at these locations appear to be necessary for an interaction of the polypeptides with phosphatidyl choline liposomes, suggesting that these residues are either involved in a direct interaction, or that the mutation affects the conformation of neighbouring amino acids that interact with the liposomes. Replacement of basic and acidic amino acids at other locations in this δ-endotoxin (boxed in Fig. 1) does not affect the toxicity or ability of the protein to bind to phosphatidyl choline liposomes (data not shown).

The model shows two α-helices (helix 1 and 2) sandwiching a β-sheet in the N-terminal portion of the protein. Helical wheel analysis (Schiffer & Edmundson, 1967) indicates that the charged residues of helix 2 are on the same side of the helix, with a rotation of 60° between each residue. Of the mutations analysed, the only mutation in this region observed to have an effect on activity is Ala124. As helix 1 appears to be protected from proteolysis when the toxin is bound to membrane vesicles (J. Horsnell, personal communication), it seems that the elimination of Lys124 in helix 2 disrupts the structural organization of the polypeptide in this region, enabling portions of the polypeptide to insert more readily into target membranes. This results in a polypeptide of increased *in-vitro* activity. The reduced *in-vivo* activity of this mutant protein, together with the ability to form only small inclusions, also suggests that it is aberrantly folded.

The spatial arrangement of the charged residues on helix 3 and 4 suggests that they might interact as shown, with intercalation of ridges and grooves on the helical surfaces as described by Chothia *et al.* (1981). This helix packing would result in the

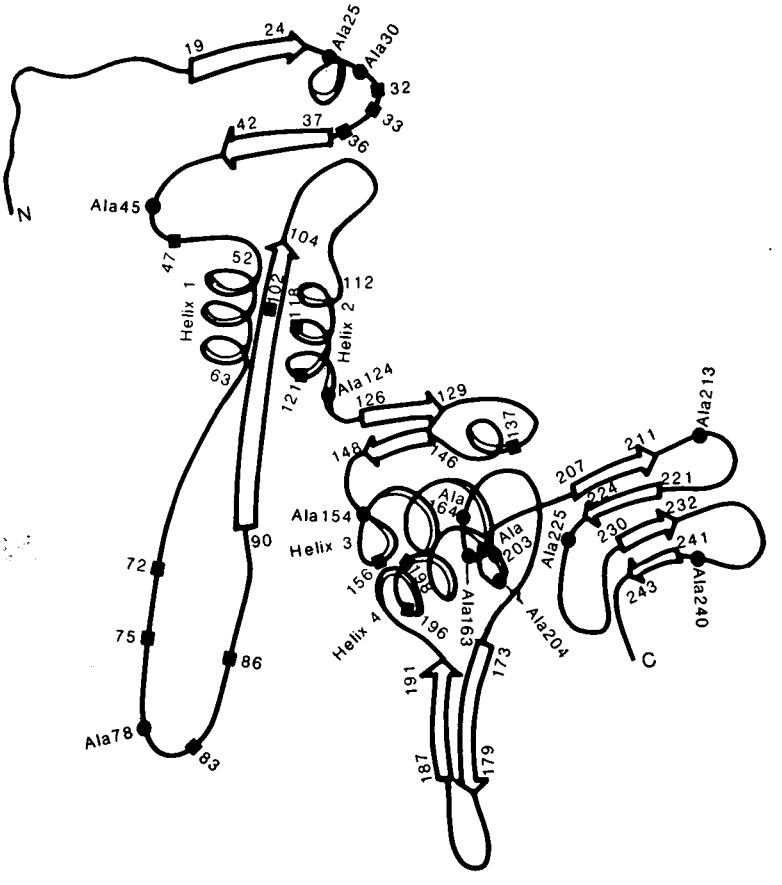

Figure 4. Secondary structural model for the *israelensis* 27,000 M_r δ-endotoxin. The mutations that have been made are indicated by the appropriate residue number. Mutations that affect the toxicity and/or expression are indicated on the polypeptide chain by filled circles; those that have no observable effect by filled squares. Predicted helical regions, β-sheet structures (open arrows) and coil/turn loops are shown.

following charge interactions: Glu164↔Lys203 and Lys163↔Glu204. Mutations of these four charged residues appear to affect both the *in-vitro* and *in-vivo* activity of the resulting polypeptides. In addition, mutation of Lys203 and Glu204 results in polypeptides which assemble into relatively small inclusions, despite a high level of synthesis. In contrast, mutation of other charged residues in these helical regions (Glu156, Glu196 and Lys198) has no detectable effect on the activity of the resulting proteins (data not shown). Alteration of Lys154 does, however, result in considerable loss of activity, suggesting that it has a key location in the overall tertiary conformation of the molecule. Evidence that the charges at residues 154 and 163 are important for the toxicity of the δ-endotoxin has been obtained by analysing the effect of mutation of Lys154 and Lys163 to arginine residues. The resulting mutant proteins retain wild-type activity (data not shown).

The data, in conjunction with the model in Figure 4, indicate that approximately one-third of the charged residues are necessary for activity and stability of the 27,000 M_r δ-endotoxin. Moreover, these essential residues are located on both helical and coil/turn regions of the predicted structural model. The elucidation of the high resolution crystallographic structure of this protein will, we hope, facilitate further interpretation of the data presented.

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