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Investigation of possible homologies between crystal proteins of three mosquitocidal strains of *Bacillus thuringiensis*

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1. SUMMARY

The possible presence of protein δ-endotoxins with homology to a cloned mosquitocidal *Bacillus thuringiensis* var. *israelensis* δ-endotoxin was investigated in two other mosquitocidal *B. thuringiensis* strains. Antiserum raised against the var. *israelensis* 27-kDa toxic polypeptide cross-reacted strongly with a 27-kDa crystal polypeptide of var. PG14 (serotype 8a : 8b, a serotype more usually associated only with lepidopteran toxicity). In addition hybridisation studies using a DNA probe derived from the cloned var. *israelensis* 27-kDa δ-endotoxin gene located a homologous region on a 72–74-MDa plasmid of PG14. A less toxic strain, var. *Kyushuensis,* showed neither immunological cross-reaction with the antiserum nor DNA hybridization with the probe.

2. INTRODUCTION

Strains of the bacterial species *Bacillus thuringiensis* produce, during sporulation, crystalline protein inclusions (δ-endotoxins) [1] that are toxic to a wide variety of insect larvae. Among the 22 serotypes that have been reported most strains are toxic to Lepidoptera [2], some to Coleoptera [3] and others, such as var. *israelensis,* var. *kyushuensis* and, a recent isolate, PG14, are toxic to dipteran larvae [4–6].

The mosquitocidal var. *israelensis* δ-endotoxin crystal comprises 4 major polypeptides of molecular weights 27 kDa, 65 kDa and a doublet of approximately 130 kDa [7]. The relative contribution of each to in vivo toxicity remains unclear and synergism or antagonism between polypeptides may be important. PG14 has been reported to have a similar polypeptide profile to var. *israelensis* except for an additional polypeptide of 144 kDa, and to have a toxicity to *Aedes aegypti* larvae very similar to that of var. *israelensis* [8]. PG14 is of further interest as it belongs to serotype 8a : 8b [6] (type strain *B. thuringiensis* var. *morrisoni*) a serotype usually associated with only lepidopteran specificity.

It has been reported that a 25-kDa polypeptide proteolytically derived from the var. *israelensis* 27-kDa polypeptide is cytolytic and larvicidal [9–11]. The gene encoding this 27-kDa polypeptide has been cloned from a 72–75-MDa plasmid [12] and protein inclusions from *B. sub-*

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tilis recombinants were cytolytic and larvicidal [13]. This paper describes the use of antibodies raised against this var. *israelensis* 27-kDa polypeptide and a probe derived from the cloned gene, to investigate the possible location of this, or closely homologous genes, in var. *kyushuensis* and PG14.

3. MATERIALS AND METHODS

3.1. Sources and growth of bacteria

B. thuringiensis var. *israelensis* IPS 78 was obtained from prof. H. de Barjac (Institute Pasteur, Paris), var. *kyushuensis* from Prof. K. Aizawai (Kyushu University, Japan), var. *morrisoni* HD12 from the Bacillus Genetics Stock Centre, Ohio State University, U.S.A. and PG14 from Dr. L.E. Padua (University of the Philippines). Var. *israelensis* IPS78-54, derived from var. *israelensis* IPS78 by curing [14], lacked the 66-MDa and 3.2-MDa plasmids of the wild type strain. Growth and sporulation were as described for *Bacillus megaterium* KM [15].

3.2. Crystal purification

Crystals were purified on discontinuous sucrose gradients as described previously [7]. Protein concentration was determined by the method of Lowry et al. [16].

3.3. SDS–PAGE and immunoblotting

Crystal proteins were analysed on 13% acrylamide gels ([17] with the modifications of Thomas and Ellar [7]) and electrophoretically transferred to nitrocellulose filters by the method of Towbin et al. [18]. Incubation with antiserum raised (as [12]) against the *israelensis* 27-kDa polypeptide and detection of bound antibodies using horseradish peroxidase conjugated anti-rabbit immunoglobulin (Miles Laboratories) were as described by Hawkes et al. [19].

3.4. Toxicity assays

Assays using mosquito larvae were conducted as described by Tyrell et al. [20] using 5-day-old *A. aegypti* larvae.

3.5. Isolation and electrophoresis of plasmids

Plasmid DNA was isolated from *B. thuringiensis* strains by a modification of the method of Casse et al. [21]. Cells were grown in CCY medium [15], harvested at mid-exponential phase, washed twice in 50 mM Tris, 20 mM EDTA pH 8.0 (TE), then resuspended in TE buffer pH 12.45 containing 3% w/v SDS and incubated at 42°C for 30 min. The solution was brought to pH 8.5–8.9 with 2 M Tris–Cl pH 7.0 and NaCl added to 3% w/v. After 30 min at room temperature the mixture was centrifuged at $7500 \times g$ for 10 min to precipitate chromosomal DNA and cell debris. The resulting supernatant was brought to 0.3 M sodium acetate, 2 vols. of 96% w/v ethanol were added and the mixture kept at −20°C overnight. The precipitated DNA was recovered by centrifugation at $7500 \times g$ for 10 min and further purified by phenol-chloroform extraction and ethanol precipitation. Plasmids were separated by electrophoresis in vertical 0.5% agarose gels and visualised under ultraviolet light after staining with ethidium bromide.

3.6. Hybridization conditions

A 1.2-kb *Tag*I fragment encoding the *israelensis* 27-kDa toxic polypeptide was purified from the 5.4-kb *EcoR*I fragment previously described [22] by the method of Dretzen et al. [23] using Schleicher and Schuell NA45 paper. The fragment was labelled to a specific activity of 1.4×10^8 c.p.m./μg DNA with [^{32}P]dATP (3000 Ci/mmol, 10 mCi/ml, Amersham International Ltd.) by nick-translation reaction [24]. An *in gel* hybridisation method based on that of Tsao et al. [25] was used. After photography, agarose gels were dried on Whatman JMM paper under vacuum at 60°C for 60 min and subsequently soaked in 0.5 M NaOH containing 0.15 M NaCl with gentle agitation for 20 min followed by 20 min in 0.5 M Tris pH 8.0, 0.15 M NaCl. Hybridisations were performed overnight at 30°c in sealed plastic bags with 50 μl of hybridisation fluid per cm^2 of dried gel. Hybridisation fluid comprised 6 × SSC [26], 5 × Denhardt's solution [27], 0.05% (w/v) sodium pyrophosphate and labelled probe at 10^6 c.p.m./ml. No pre-hybridisation was necessary. Gels were subsequently washed in two changes of

6 × SSC, 0.05% (w/v) sodium pyrophosphate. The wash temperature was varied (based on the calculations of [28] and [29] and a 33 mol% G-C content of the probe [13]) to obtain the required stringency. Gels were then air dried and autoradiographed at −80 °C for 1–6 days.

4. RESULTS AND DISCUSSION

Purified crystals of both *israelensis* and PG14 gave an LC_{50} for *A. aegypti* larvae of approx. 15 ng/ml after 60 min and less than 1 ng/ml after 24 h. *Kyushuensis* crystals were significantly less toxic with LC_{50} values of more than 1 μg/ml after 60 min and approx. 15 ng/ml after 24 h. The immunoblot (Fig. 1b) shows a strong cross-reaction between the PG14 27-kDa polypeptide and the *israelensis* 27-kDa antiserum, indicating protein homology between the two polypeptides. No such cross-reaction was observed with *kyushuensis* although this does not preclude homology between other polypeptides in the crystals. The plasmid profiles of the strains (Fig. 2a) reveal that PG14, like *israelensis,* possesses a plasmid of approx. 74 MDa (in contrast to a previous report [8]). This plasmid hybridised with the *israelensis* 27-kDa δ-endotoxin gene probe under conditions allowing up to 30% mismatch, indicating the presence of a homologous region. No hybridisation of the probe to the *kyushuensis* DNA was observed, even under conditions of low stringency (35% mismatch). The 25–26-kDa polypeptide of *kyushuensis* may therefore represent a separate class of crystal protein.

PG14 has been serotyped as 8a:8b [6]; the type-strain of this serotype, var. *morrisoni* HD12, exhibits no toxicity to mosquitoes (Padua et al., unpublished and [6]), produces only a P-1 type [30] polypeptide complex (Fig. 1a) and has no plasmids in common with PG14 (data not shown).

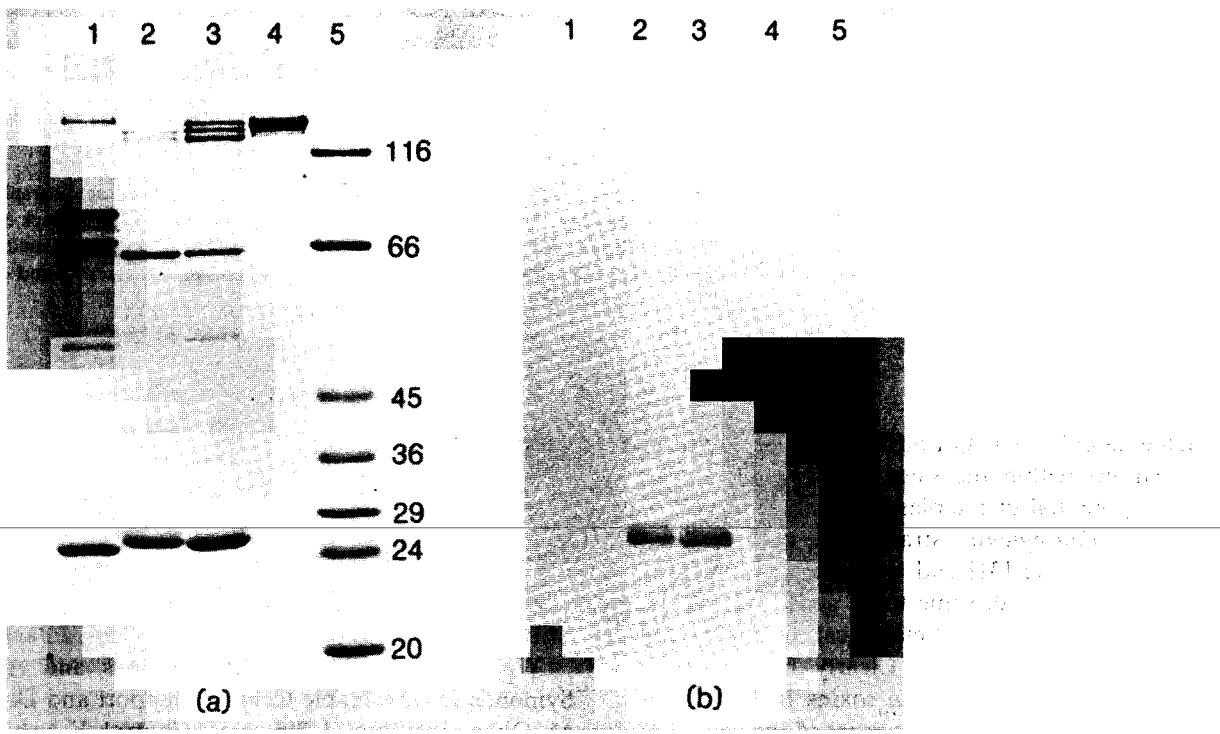

Fig. 1. (a) SDS/13% polyacrylamide gel electrophoresis, Coomassie blue stained, of crystal proteins, and (b) immunoblot using antiserum as described. Track 1, *kyushuensis*; track 2, *israelensis* IPS78; track 3, PG14; track 4, *morrisoni* HD-12; track 5, molecular mass standards (kDa).

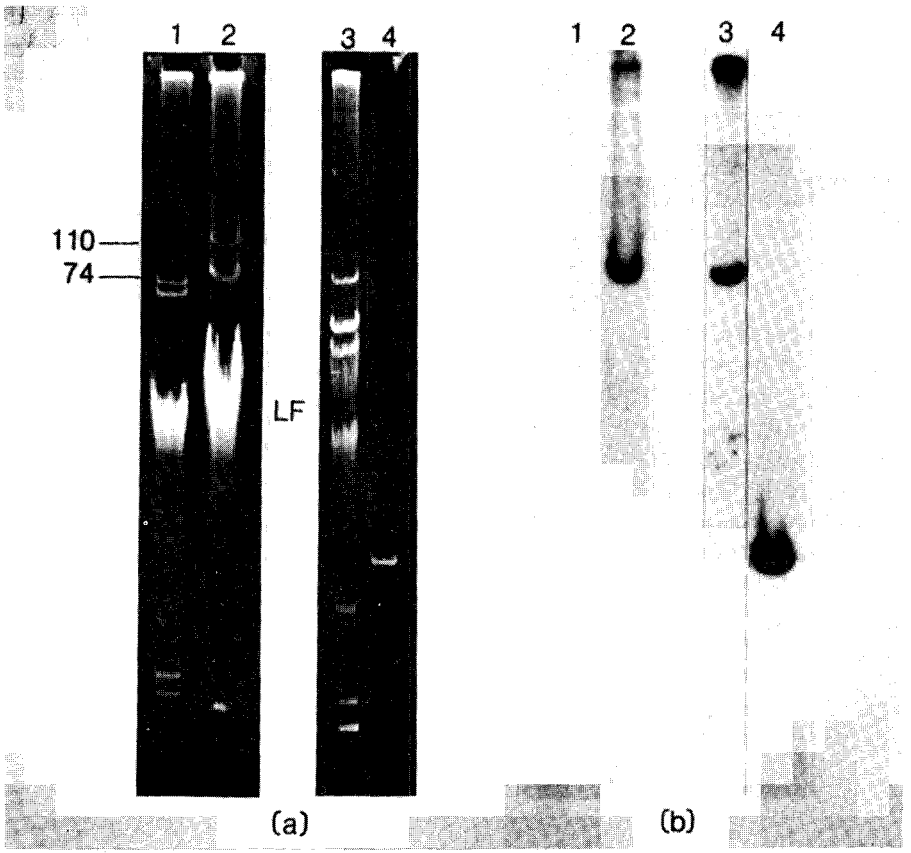

Fig. 2. (a) 0.5% agarose gel electrophoresis, ethidium bromide stained, of plasmids of *B. thuringiensis* strains, and (b) autoradiograph resulting from in gel hybridisation using ^{32}P-labelled probe as described. Track 1, *kyushuensis*; track 2, *israelensis* IPS78-54; track 3, PG14; track 4, cloned *israelensis* 27-kDa δ-endotoxin gene. Left-hand margin figures refer to sizes of *israelensis* IPS78-54 plasmids (MDa), LF denotes linear DNA fragments. (b) Tracks 1 and 2 produced under conditions allowing 35% mismatch, tracks 3 and 4 under conditions allowing 30% mismatch.

Such data indicate major differences between these two strains within the same serotype. Given the coding potential of the plasmid content typical of many *B. thuringiensis* strains, the possibility of conjugal transfer [31] and the transposition function associated with some toxin genes [32,33], the utility of this serological classification for toxicity studies is probably limited. Clearly the occurrence and origin of mosquitocidal toxins in PG14 and possible transpositional properties of the genes are of interest. A gene encoding a 27-kDa polypeptide has now been cloned from the PG14 74-MDa plasmid. DNA sequence data have shown this gene to be highly homologous to the *israelensis* 27-kDa gene (manuscript in preparation).

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