Gene, 52 (1987) 285–290
Elsevier

GEN 01937

Cloning and heterologous expression of an insecticidal delta-endotoxin gene from *Bacillus thuringiensis* var. *aizawai* IC1 toxic to both lepidoptera and diptera

(Recombinant DNA; *Escherichia coli*; plasmid vector; gene probe; P1 toxins)

M.Z. Haider, E.S. Ward and D.J. Ellar

Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW (U.K.)

Received 2 October 1986
Revised 15 December 1986
Accepted 19 December 1986

SUMMARY

Bacillus thuringiensis var. *aizawai* IC1 synthesises an insecticidal protein δ-endotoxin (130–135 kDa) that is toxic to both lepidopteran and dipteran larvae, and cross-reacts immunologically with certain monospecific lepidopteran toxins. A 166-kb plasmid from this bacterium was found to hybridise with an intragenic probe derived from the cloned *B. thuringiensis* var. *sotto* lepidopteran-specific δ-endotoxin gene. A strongly hybridising 5.2-kb *Sst*I fragment from var. *aizawai* plasmid DNA was cloned in pUC18. After subcloning of this DNA in *Escherichia coli*, recombinants were obtained that synthesised large amounts of a 130–135-kDa protein. The protein was deposited in the cytoplasm as microscopically visible inclusion bodies and lysates of these cells were found to be toxic to both lepidopteran and dipteran larvae by comparison with controls. The structural basis for the dual specificity of this var. *aizawai* toxin is now amenable to further study.

INTRODUCTION

Strains of *B. thuringiensis* produce crystalline protein δ-endotoxins that are toxic to a variety of insect larvae (Luthy, 1980). The δ-endotoxins are synthesised during the sporulation phase of growth as parasporal inclusions (Bulla et al., 1980; Somerville, 1978). Many *B. thuringiensis* strains possess high M_r P1-type polypeptides (130–135 kDa; Yamamoto and McLaughlin, 1981), which are immunologically related (Ellar et al., 1986a). Some of these strains also show mosquitocidal activity that was thought to be associated exclusively with a protein of 63-kDa (P2; Yamamoto and McLaughlin, 1981). Recently, however, strains in which the crystal δ-endotoxin consists entirely of P1-type proteins have been shown to possess mosquitocidal activity in addition to being toxic in vivo to *Lepidoptera* and to cells from diverse lepidopteran groups in vitro (Haider et al., 1986a,b; Knowles et al., 1986). In one such strain deposited in a culture collection as *B. thuringiensis* var. *colmeri*, but subsequently found by serotyping to be a var. *aizawai* strain (see below), the specificity for different insects was found to be the result of differential proteolysis of a single P1-type protoxin by

Correspondence to: Dr. D.J. Ellar, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW (U.K.) Tel. (0223)333651.

Abbreviations: bp, base pair(s); EtdBr, ethidium bromide; IPTG, isopropyl-β-D-thiogalactoside; kb, 1000 bp; SDS, sodium dodecyl sulphate; [ ], designates plasmid-carrier state.

larval gut proteases (Haider et al., 1986a). Cleavage of approx. 30 amino acids from the lepidopteran-specific form of the toxin was sufficient to convert it to a form toxic only to diptera (Haider et al., 1986a). The sequence of this protein therefore offers a unique opportunity to investigate the specificity determinants of these δ-endotoxins and this report describes the cloning and expression of this gene as a first step to obtaining the amino acid sequence.

B. thuringiensis δ-endotoxin genes have been cloned variously from high-M_r plasmids or chromosomal DNA (Schnepf and Whiteley, 1981; Klier et al., 1982; Shibano et al., 1985; Adang et al., 1985; Honigman et al., 1986; Sekar and Carlton, 1985; Ward et al., 1984). In most cases the δ-endotoxin genes have been isolated from high-M_r plasmids although there are exceptions (Honigman et al., 1986; Held et al., 1982; Klier et al., 1982). Kronstad and Whiteley (1983) have reported the diversity of location of a δ-endotoxin gene encoding a P1-type toxin. Using a cloned var. *kurstaki* δ-endotoxin gene as a hybridisation probe, homologous genes in different *B. thuringiensis* strains have been found (Kronstad and Whiteley, 1983). All the above reports describe δ-endotoxin genes whose product is toxic to only one insect group. Because the dual specificity *aizawai* toxin cross-reacts immunologically with several of these lepidopteran toxins, sequence comparisons will be particularly interesting.

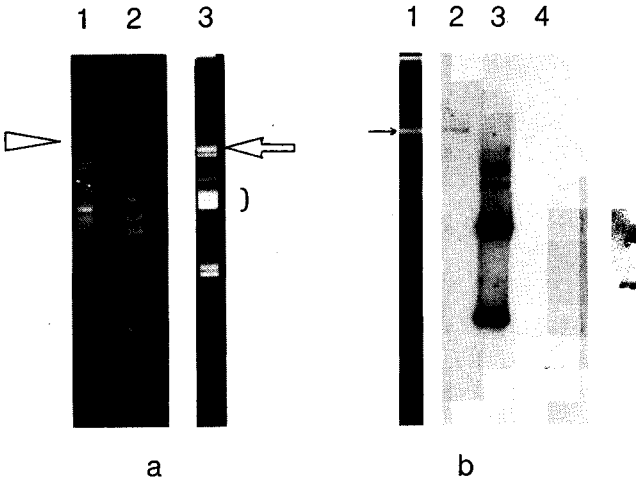

Fig. 1. Agarose gel (0.5%) electrophoresis of plasmids from *B. thuringiensis* strains isolated by a lysozyme-detergent extraction procedure based on that of Birnboim and Doly (1979). (**a**) Lanes: 1, *B. thuringiensis* var. *thuringiensis* HD-2; 2, var. *kurstaki* HD-1; 3, var. *aizawai* IC1. (**b**) Lanes: 1 and 2, purified high-M_r plasmids of var. *aizawai* IC1 and an autoradiogram showing hybridisation of a high-M_r plasmid in the 166-kb region with the var. *sotto* δ-endotoxin gene probe (Shibano et al., 1985); a 2.8-kb *Nsi*I-*Nru*I fragment isolated by the method of Dretzen et al. (1981) was radiolabelled using random oligodeoxynucleotide primers (Feinberg and Vogelstein, 1983) and hybridised by a modification (Earp et al., 1987) of the method of Tsao et al. (1983); 3, covalently closed circular pSE2 (the plasmid harbouring var. *sotto* gene); 4, phage λ *Hin*dIII standards. The position of chromosomal smear is indicated by a bracket. The open arrowhead on the left margin points to the 180-kb plasmid of var. *thuringiensis* HD-2. The arrow (in **a** and **b**) indicates 166-kb plasmid of var. *aizawai* IC1. The gels (**a**, lanes 1–3; **b**, lane 1) were stained with EtdBr (0.5 μg/ml) in water for 30 min and photographed.

EXPERIMENTAL AND DISCUSSION

(a) *Bacillus thuringiensis* var. *aizawai* IC1 and its plasmids

The *B. thuringiensis* strain used in this study was originally obtained as *B. thuringiensis* var. *colmeri*, HD-847 from the Bacillus Genetics Stock Centre (Ohio State University, Columbus, OH, U.S.A.), and was described as such in the reports by Haider et al. (1986a,b), Ellar et al. (1986a,b). When the serotype of this strain was checked by Dr. M. Ohba (Institute of Biological Control, Faculty of Agriculture, Kyushu University, Fukuoka, Japan) it was found to be serotype 7 (var. *aizawai*) and not serotype 21 (var. *colmeri*) as listed in the catalogue of the Bacillus Genetics Stock Centre. The strain will therefore be described as *B. thuringiensis* var. *aizawai* IC1 in this work and any subsequent publications. The patterns of plasmids isolated from *B. thuringiensis* var. *aizawai* IC1, var. *kurstaki* HD-1 and var. *thuringiensis* HD-2 are shown in Fig. 1a. The plasmids isolated from *B. thuringiensis* var. *aizawai* range from 14–180 kb. The high-M_r plasmid(s) from *B. thuringiensis* var. *aizawai* IC1 were purified by preparative vertical electrophoresis in 3.0 mm thick 0.5% low-gelling-temperature agarose slabs (Seaplaque, FMC Colloids; Weislander, 1979). A plasmid in the region of 166-kb hybridised with the var. *sotto* δ-endotoxin gene probe (Fig. 1b). This suggested the presence of a homologous δ-endotoxin gene on a plasmid of approximately this size.

(b) Cloning of δ-endotoxin gene and expression in *Escherichia coli*

Digestion of total plasmid DNA of *B. thuringiensis* var. *aizawai* IC1 with various restriction enzymes revealed several fragments that hybridised with the var. *sotto* δ-endotoxin gene probe. A 5.2-kb *Sst*I fragment which hybridised strongly was cloned in *Sst*I-cleaved pUC18 (Yanisch-Perron et al., 1985), using *E. coli* TG1 (Gibson, 1984) as host. The plasmids harbouring the required inserts were identified by hybridization of isolated DNA with the ^{32}P-labelled probe (Fig. 1, legend). The DNA from these recombinants was used to prime protein synthesis in the *E. coli* cell-free system (Chen and Zubay, 1983; Bottomly and Whitfeld, 1979). A polypeptide was specifically immunoprecipitated from this system, using var. *kurstaki* P1 antiserum (Howe et al., 1982; Haider et al., 1986a). A simple restriction map of the recombinant plasmid (pIC5, containing the 5.2-kb *Sst*I fragment) is presented in Fig. 2. The location of the δ-endotoxin gene on the insert was determined by studying δ-endotoxin gene expression in the *E. coli* cell-free system primed with pIC5 DNA cleaved with *Cla*I, *Hin*dIII or *Pst*I. The results obtained (not shown) indicate that *Hin*dIII sites are located within the structural gene, whereas *Cla*I and *Pst*I cleave the fragment extragenically. Therefore, a 4-kb *Sst*I-*Cla*I fragment derived from pIC5 was subcloned in *Sst*I + *Acc*I-cleaved vector to generate the plasmid pIF2 (Fig. 2b). Recombinant *E. coli* cells harbouring plasmid pIF2 were grown to late stationary phase in the presence of the *lacZ* inducer IPTG, at which time distinct phase-bright inclusions were detected by phase-contrast microscopy. The inclusions were not present in cells carrying pUC18 vector nor in the original pIC5 recombinants. This suggests that in pIF2 the *Sst*I-*Cla*I fragment contains a δ-endotoxin gene transcribed from the *lacZ* promoter of the vector. This would account for the higher level of expression in *E. coli* TG1[pIF2] cells compared to TG1[pIC5], in which the insert is in the opposite orientation with respect to the *lacZ* promoter.

A second subclone was constructed by cleaving pIF2 with *Pst*I, followed by ligation and screening for recombinants with 3.65-kb inserts which hybridised with the var. *sotto* gene-probe (pIH5; Fig. 2). These cells produce phase-bright inclusions when grown as described earlier. The in vivo synthesis of δ-endotoxin by recombinant *E. coli* cells carrying pIH5 was analysed using polyacrylamide gel electrophoresis of cell lysates and immunoblotting with var. *kurstaki* P1-antiserum (Fig. 3). A 130–135-kDa protein was clearly detectable in the lysates of recombinant pIH5 cells which also crossreacted with P1 antiserum (Fig. 3, lane 3) and was absent in the control cell lysates (Fig. 3, lane 2).

The results of toxicity tests conducted on the lepidopteran and dipteran larvae are summarised in Table I. *Aedes aegypti* larvae fed with lysates of pIH5 cells showed symptoms of toxicity within 6 h, whereas the control larvae were unaffected even after 24 h. In the case of the *Pieris brassicae* larvae, rapid cessation of feeding was observed, with 50% larval mortality occurring 20 h after initiation of the assay.

TABLE I

Toxicity to *Pieris brassicae* and *Aedes aegypti* larvae of (i) the lysates prepared from recombinant *Escherichia coli* cells and of (ii) δ-endotoxin crystals of *Bacillus thuringiensis* var. *aizawai* IC1 [a]

	Proportion of *P. brassicae* larvae dead after			Proportion of *A. aegypti* larvae dead after	
	3h	6h	24h	6h	24h
E. coli [pUC18]	0	0	0	0	0
E. coli [pIF2]	0	2/5	4/5	15/25	25/25
E. coli [pIH5]	0	0	2/5	13/25	22/25
B. thuringiensis var. *aizawai* IC1 crystals (10 μg ml)	0	4/5	5/5	25/25	25/25

[a] The cells were washed once with 0.1 M NaCl and lysed by sonication (2 bursts of 30 s each) with a sonic probe (Dawe Instruments) at maximum intensity. The assay procedures have been described earlier (Thomas and Ellar, 1983; Haider et al., 1986). *B. thuringiensis* δ-endotoxin crystals were purified by differential ultracentrifugation through sucrose gradients (Haider et al., 1986a).

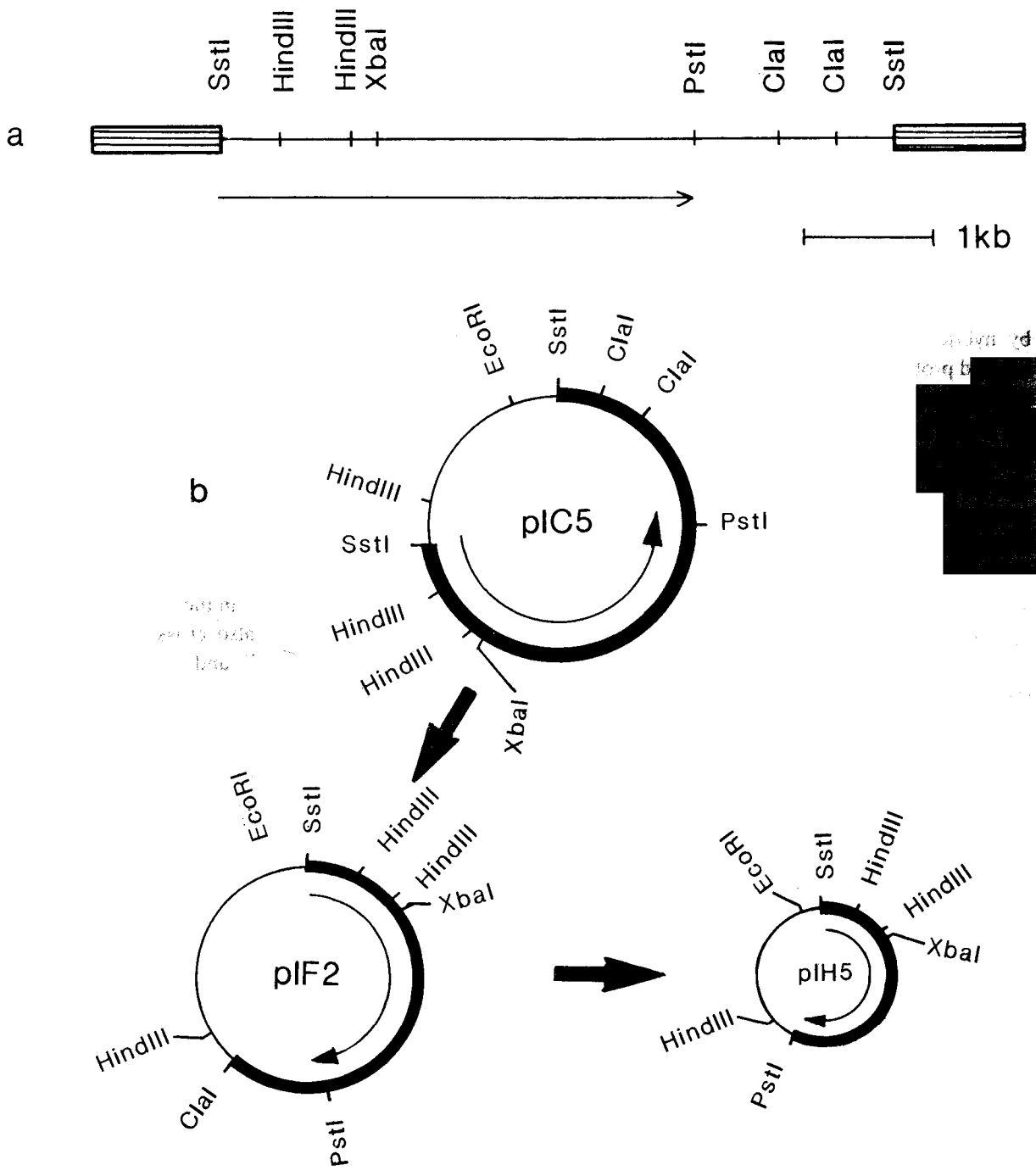

Fig. 2. Plasmids with δ-endotoxin gene. **(a)** Restriction map of the 5.2-kb insert of pIC5. Shaded areas represent pUC18 vector DNA; **(b)** construction of plasmids pIF2 and pIH5 from plasmid pIC5 [restriction map presented in **(a)**] containing the cloned *B. thuringiensis* var. *aizawai* IC1 δ-endotoxin gene. Thick lines represent var. *aizawai* IC1 DNA and the arrows indicate location and orientation of the gene determined by analysis of subclones in the *E. coli* cell-free system. See RESULTS, section **b**, for further details.

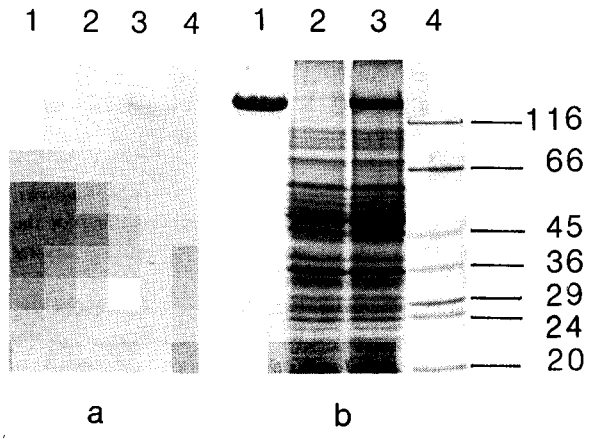

Fig. 3. Immunological detection of the cloned gene product in the cell lysates prepared from recombinant *E. coli* cells. The cells were induced with 1 mM IPTG immediately before the onset of stationary phase and lysed by the procedure of Shivakumar et al. (1986); the lysates were electrophoresed on SDS–13% polyacrylamide gel (Laemmli, 1970) and immunoblotted with var. *kurstaki* P1 antiserum and the bound antibody was detected using horse-radish peroxidase conjugated anti-rabbit immunoglobulin (Towbin et al., 1979; Hawkes et al., 1982). (**a**) Immunoblot with P1 antiserum. Lanes: 1, purified crystal protein from var. *aizawai* IC1; 2, *E. coli* TG1 [pUC18] lysate; 3, *E. coli* [pIH5] lysate; 4, M_r standards (Sigma). (**b**) Coomassie blue-stained gel of the same samples as in panel (**a**). Numbers on the right margin are in kDa, and refer to lane 4 in (**b**).

(c) Conclusions

(1) An insecticidal δ-endotoxin gene has been cloned in *E. coli*. The hybridization experiments suggest that this gene is located on a high-M_r plasmid of *B. thuringiensis* var. *aizawai* IC1.

(2) The gene is expressed in *E. coli* when aligned with the vector *lacZ* promoter. This suggests that the native *B. thuringiensis* promoter is either absent or poorly recognised in *E. coli*. Alternatively there may be regulatory sequences flanking the var. *aizawai* IC1 gene which may have been deleted during construction of pIF2 and pIH5 using pIC5 DNA.

(3) Toxicity studies using cell extracts of recombinant *E. coli* revealed that the cloned gene product is toxic to both lepidopteran and dipteran larvae.

Determination of the nucleotide sequence of the *B. thuringiensis* var. *aizawai* IC1 gene will hopefully aid in our understanding of the evolution of these P1 type toxins and the structural determinants of their lepidopteran specificity.

ACKNOWLEDGEMENTS

This work was supported by the Royal Commission for the Exhibition of 1851, the Agricultural and Food Research Council. E.S.W. is a Research Fellow of Gonville and Caius College, Cambridge. We thank Dr. K.F. Chak and Mr. D.J. Earp for their helpful advice, T.J. Sawyer, C. Nicholls and Mrs. Audrey Symonds for their technical assistance and Mr. D. Funnel for supplying *Aedes aegypti* eggs.

REFERENCES

Adang, M.J., Staver, M.J., Rocheleau, T.A., Leighton, J., Baker, R.F. and Thompson, D.V.: Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. Gene 36 (1985) 289–300.

Birnboim, H.C. and Doly, J.: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. 7 (1979) 1513–1523.

Bottomley, W. and Whitfeld, P.R.: Cell-free transcription and translation of total spinach chloroplast DNA. Eur. J. Biochem. 93 (1979) 31–39.

Bulla Jr., L.A., Bechtel, D.B., Kramer, K.J., Shethna, Y.I., Aronson, A.I. and Fitz-James, P.C.: Ultrastructure, physiology and biochemistry of *Bacillus thuringiensis*. Crit. Rev. Microbiol. 8 (1980) 147–204.

Chen, H-Z. and Zubay, G.: Prokaryotic coupled transcription-translation. Methods Enzymol. 101 (1983) 674–690.

Dretzen, G., Bellard, M., Sassone-Corsi, P. and Chambon, P.: A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. 112 (1981) 295–298.

Earp, D.J., Ward, E.S. and Ellar, D.J.: Investigation of possible homologies between crystal proteins of three mosquitocidal strains of *Bacillus thuringiensis*. FEMS Microbiol. Lett. (1987) in press.

Ellar, D.J., Knowles, B.H., Haider, M.Z. and Drobniewski, F.A.: Investigation of the specificity, cytotoxic mechanisms and relatedness of *Bacillus thuringiensis* insecticidal δ-endotoxins from different pathotypes. In Falmagne, P. Alouf, J.E., Fehrenbach, F.J., Jeljaszewicz, J. and Thelestam, M. (Eds.), Bacterial Protein Toxins. G. Fischer, Stuttgart, 1986a, pp. 44–48.

Ellar, D.J., Knowles, B.H., Drobniewski, F.A. and Haider, M.Z.: The insecticidal specificity and toxicity of *Bacillus thuringiensis* δ-endotoxins may be determined respectively by an initial binding to membrane-specific receptors followed by a common mechanism of cytolysis. In Samson, R.A., Vlak, J.M. and Peters, D. (Eds.), Fundamental and Applied Aspects of Invertebrate Pathology. Foundation of the Fourth International Colloquium of Invertebrate Pathology, Wageningen (The Netherlands), 1986b, pp. 7–10.

Feinberg, A.P. and Vogelstein, B.: A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132 (1983) 6–13.

Gibson, T.: Studies on the Epstein–Barr Virus Genome. Ph.D. Thesis, University of Cambridge, Cambridge (U.K.) 1984.

Haider, M.Z., Knowles, B.H. and Ellar, D.J.: Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal δ-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. Eur. J. Biochem. 156 (1986a) 531–540.

Haider, M.Z., Knowles, B.H. and Ellar, D.J.: A role for gut enzymes in determining the in vitro specificity of *Bacillus thuringiensis* var. *colmeri* δ-endotoxin. In Falmagne, P. Alouf, J.E., Fehrenbach, F.J., Jeljaszewicz, J. and Thelestam, M. (Eds.), Bacterial Protein Toxins. G. Fischer, Stuttgart, 1986b, pp. 59–60.

Hawkes, R., Niday, E. and Gordon, J.: A dot immunobinding assay for monoclonal and other antibodies. Anal. Biochem. 119 (1982) 142–147.

Held, G.A., Bulla, Jr., L.A., Ferrari, E., Hoch, J., Aronson, A.I. and Minnich, S.A.: Cloning and localization of the lepidopteran protoxin gene of *Bacillus thuringiensis* subsp. *kurstaki*. Proc. Natl. Acad. Sci. USA 79 (1982) 6065–6069.

Honigman, A., Nadjar-Pazerini, G., Yawetz, A., Oron, U., Schuster, S., Broza, M. and Sneh, B.: Cloning and expression of the lepidopteran toxin produced by *Bacillus thuringiensis* var. *thuringiensis* in *Escherichia coli*. Gene 42 (1986) 69–77.

Howe, C.J., Bowman, C.M., Dyer, T.A. and Gray, J.C.: Localization of wheat chloroplast genes for beta and epsilon subunits of ATP synthase. Mol. Gen. Genet. 186 (1982) 525–530.

Klier, A., Fargette, F., Ribier, J. and Rapoport, G.: Cloning and expression of the crystal protein genes from *Bacillus thuringiensis* strain *berliner* 1715. EMBO J. 1 (1982) 791–799.

Knowles, B.H., Francis, P.H. and Ellar, D.J.: Structurally related *Bacillus thuringiensis* δ-endotoxins display major differences in insecticidal activity in vivo and in vitro. J. Cell Sci. 84 (1986) 221–236.

Kronstad, J.W., Schnepf, H.E. and Whiteley, H.R.: Diversity of locations for *Bacillus thuringiensis* crystal protein genes. J. Bacteriol. 154 (1983) 419–428.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (1970) 680–685.

Lüthy, P.: Insecticidal toxins of *Bacillus thuringiensis*. FEMS Microbiol. Lett. 8 (1980) 1–7.

Schnepf, H.E. and Whitely, H.R.: Cloning and expression of the *Bacillus thuringiensis* crystal protein in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 78 (1981) 2893–2897.

Sekar, V. and Carlton, B.C.: Molecular cloning of the δ-endotoxin gene of *Bacillus thuringiensis* var. *israelensis*. Gene 33 (1985) 151–158.

Shibano, Y., Yamagata, A., Nakamura, N., Iizuka, H., Sugisaki, H. and Takanami, M.: Nucleotide sequence coding for the insecticidal fragment of the *Bacillus thuringiensis* crystal protein. Gene 34 (1985) 243–251.

Shivakumar, A.G., Gundling, G.J., Benson, T.A., Casuto, D., Miller, M.F. and Spear, B.B.: Vegetative expression of the δ-endotoxin genes of *Bacillus thuringiensis* subsp. *kurstaki* in *Bacillus subtilis*. J. Bacteriol. 166 (1986) 194–204.

Somerville, H.J.: Insect toxin in spores and protein crystal of *Bacillus thuringiensis*. Trends Biochem. Sci. 3 (1978) 108–110.

Thomas, W.E. and Ellar, D.J.: *Bacillus thuringiensis* var. *israelensis* crystal δ-endotoxin: Effects on insect and mammalian cells in vitro and in vivo. J. Cell Sci. 60 (1983) 181–197.

Towbin, H., Staehelin, T. and Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose sheets: procedure and simple applications. Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.

Tsao, S.G.S., Brunk, C.F. and Pearlman, R.E.: Hybridization of nucleic acids directly in agarose gels. Anal. Biochem. 131 (1983) 365–372.

Ward, E.S., Ellar, D.J. and Todd, J.A.: Cloning and expression in *Escherichia coli* of the insecticidal δ-endotoxin gene of *Bacillus thuringiensis* var. *israelensis*. FEBS Lett. 175 (1984) 377–382.

Weislander, L.: A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. Anal. Biochem. 98 (1979) 305–309.

Yamamoto, T. and McLaughlin, R.E.: Isolation of a protein from the parasporal crystal of *Bacillus thuringiensis* var. *kurstaki* toxic to the mosquito larva, *Aedes taeniorhincus*. Biochem. Biophys. Res. Commun. 103 (1981) 414–421.

Yanisch-Perron, C., Vieira, J. and Messing, J.: Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19. Gene 33 (1985) 103–119.

Communicated by K.F. Chater.