J. Fac. Appl. Biol. Sci.,
Hiroshima Univ. (1989), 28 : 63—69

Cloning and Partial Characterization of the *Bacillus thuringiensis dendrolimus* T84A1 Insecticidal Crystal Protein Gene

Jiro SHIBATA, Masakatsu TAMIYA and Yasunori NAGAMATSU

Department of Applied Biochemistry, Faculty of Applied Biological Science, Hiroshima University, Higashi-Hiroshima 724, Japan

Recieved September 1, 1989

Abstract *Pst* I -fragments from three plasmid DNAs (10.0, 19.3 and 51.1 kb) of *Bacillus thuringiensis* (*Bt*) *dendrolimus* T84A1 were cloned in *Escherichia coli*. One of transformants, which carrys a 10.22 kb *Bt* DNA (9.91 and 0.31 kb *Pst* I -segments), produced a 145 k polypeptide which has the same electrophoretic mobility as the crystal protein (CP) and reacts with anti-CP antibodies. Deletion analysis revealed that the entire CP gene resided on the 5.0 Kb *Hpa* I -*Pst* I region within the 9.91 kb *Pst* I -segment. Hybridization analysis showed that the cloned gene was derived from the largest plasmid and might be the only one in this *Bt* strain. The restriction enzyme map of the cloned segment was very similar to it of the *sotto* strain except the 5′-adjacent region of the CP gene.

INTRODUCTION

Various strains among 34 subspecies of *Bacillus thuringiensis* (*Bt*) have been isolated which produce the crystal proteins toxic to lepidopteran, dipteran or coleopteran insects. Since the first cloning of the crystal protein (CP) gene from a plasmid in the *kurstaki* strain (SCHNEPF and WHITELEY, 1981), a number of CP genes have been isolated from various strains, and the nucleotide sequences were determined (SCHNEPT *et al.*, 1985; SHIBANO *et al.*, 1986; KONDO *et al.*, 1987; SCHIMIZU *et al.*, 1988). These studies revealed some extent of structural variations of the genes and also the existence of multiple genes in a strain. Significant differences in insecticidal specificity were seen even within subspecies producing lepidopteran toxins (HÖFTE *et al.*, 1988). Insect specificity should be related to both the crystal protein structure and some property of the insect. An approach for this subject using protein engineering techniques was reported recently (ALBERT *et al.*, 1989). In the present study, we cloned a crystal protein gene from *Bacillus thuringiensis dendrolimus* T84A1 which is pathogenic to lepidopteran insects.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Bacillus thuringiensis* subsp. *dendrolimus* T84A1 is a kind gift from Prof. Aizawa, Kyushu University. For cloning experiments, plasmid vector pBR322 was used with *E. coli* strain χ1776 or HB101, and vector pUC119 with *E. coli* MV1184.

Preparation and analysis of plasmids and total cell DNA. Plasmid DNAs were ex-

tracted and partially purified by the alkaline method of BIRNBOIM (1983) without lysozyme for *Bt.* DNA solutions obtained were treated with phenol in the presence of 0.3% SDS and added with ammonium acetate (up to 2.4 M) to precipitate the bulk of RNAs. After treatment with ribonuclease A and T_1, plasmid DNAs were isolated by ethidium bromide (EtBr)-CsCl gradient centrifugation. Total cell DNA was prepared according to KRONSTAD *et al.* (1983). Electrophoretic analysis of plasmids and their fragments was conducted in 36 mM Tris-30 mM NaH_2PO_4-1 mM EDTA·Na_2 with 0.6% agarose gel.

Preparation and purification of antibodies. The insecticidal tryptic fragment (Fragment T) of the crystal protein from the *dendrolimus* T84A1 strain was prepared and purified as described previously (NAGAMATSU *et al.*, 1984) and used as an antigen to immunize rabbits. The monospecifc antibody was purified by affinity chromatography of the antiserum on a Fragment T-Sepharose 4B column. The antibody reacted with either Fragment T or the crystal protein, that was judged by immunoelectrophoresis.

Western blotting and enzyme immunodetection of polypeptide antigens. Whole cell extracts of *E. coli* transformants were prepared in the presence of SDS and urea according to Schnepf and WHITELEY (1985), and subjected to SDS-polyacrylamide gel electrophoresis. Proteins in the gels were electrotransferred to nitrocellulose filters in 25 mM Tris-glycine buffer, pH 8.3. The filters were blocked with bovine serum albumin and succesively reacted with anti-Fragment T antibody and anti-rabbit IgG labeled with horseradish peroxidase (Bio-Rad). Polypeptide bands showing antigenicity were developed in 0.05% 3, 3′-diaminobenzidine-0.01% H_2O_2, pH 7.2. Mild extraction of antigens was carried out as follows: cells were suspended in 50 mM Tris-5 mM EDTA-HCl, pH 7.5 and disrupted by sonication at 30 W for 10 min in an ice-bath. After centifugation at 12,000×g for 1 hr, antigens were extracted from the insoluble material with sodium carbonate (Na^+; 0.1 M) buffer, pH 10.2, containing 20 mM dithiothreitol at 0°C.

Southern hybridization. After agarose gel electrophoresis, DNAs in the gel were partially cleaved in 0.25 N HCl for 15 min, denatured and transferred to Hybond-N nylon filter (Amarsham) by the method of SOUTHERN (1975). Preparation of labeled probes, hybridization and detection were carried out with Nonradioactive DNA labeling and Detection Kit (Boehringer Mannheim Biochemicals) according to the supplier's mannual.

Other methods. DNA ligation and transformation of *E. coli* were carried out as described in the mannual of MANIATIS *et al.* (1982).

RESULTS

As shown in Fig. 1A, six DNA bands, the three major (pA, pB and pC) and the three minor (A′, B′ and C′), were detected by agarose gel electrophoresis of total plasmid DNA preparation from *Bt dendrolimus* T84A1. The three minor DNAs were often absent after EtBr-CsCl gradient centrifugation, and showed different sedimentation behaviors on a 5-25% sucrose gradient from them of the three major DNAs (Fig. 1B), indicating that they were different (linear or open circular) forms derived from the three major cccDNAs. From a calibration curve with cccDNA or linear DNA size makers, the sizes of pA and pB were estimated as 10.0 and 19.3 kb, respectively, and A′ and B′ DNAs were confirmed to be linear forms of pA and pB, respectively. The largest plasmid (pC) gave three *BamH* I

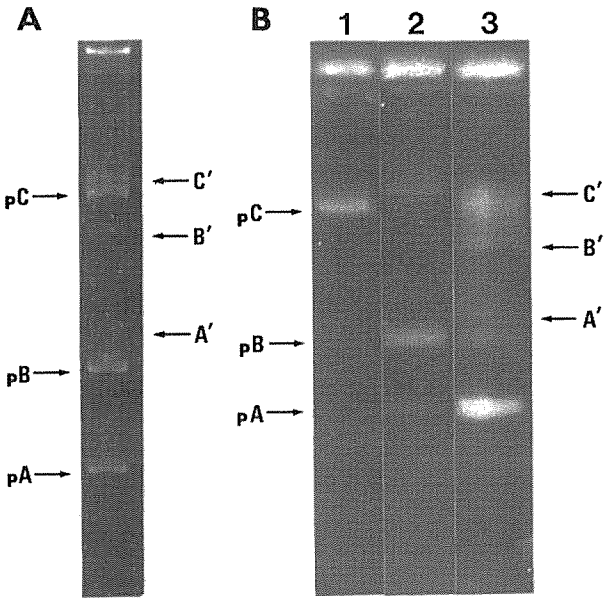

Fig. 1. Agarose gel electrophoretic analysis of plasmid DNAs from *B. thuringiensis dendrolimus* T84A1 in 0.6% gels. (A) Total plasmid complement. (B) Plasmid fractions separated by sucrose density gradient centrifugation. 1, "bottom (fast) fraction"; 2, "middle (medium) fraction"; 3, "top (slow) fraction".

-fragments (11.4, 18.5 and 21.2 kb) or two *Sal* I -fragments (28 and 23.2 kb), thus the size was calculated as 51.1 kb.

Cloning of the crystal protein (CP) gene was performed by insertion of *Pst* I -fragments from total *Bt* plasmid DNAs into pBR322 and transfomation of *E. coli*. One colony, among 41 tetracyclin-resistant and ampicillin-sensitive transformants, produced a 145 k protein reactive with the monospecific antibodies to the insecticidal fragment prepared form the crystal protein, as judged by Western blotting and enzyme immunodetection (Fig. 2). The recombinant plasmid carried by the colony was designated pT509.

The recombinant plasmid pT 509 has a 10.22 kb insert (9.91 and 0.31 kb *Pst* I -segments), and its restriction enzyme map is shown in Fig. 3, top. To determine the location of the CP gene on the insert of pT509, deletion derivatives were constructed as shown in Fig. 3 and introduced into *E. coli*. The transformant harboring pT509ΔK4 lost productivity of the crystal protein (140 k antigen). The 5 kb *Hpa* I -*Pst* I segment was subcloned into pBR322 using a *Pst* I linker. *E. coli* carring the plasmid pHP206 (the 0.31 kb *Pst* I -segment remained) prduced 145 k antigen. The orientation of insertion of the 5 kb segment did not affect the antigen productivity, indicating that the entire CP gene including promoter and terminator risided on the 5.0 kb *Hpa* I -*Pst* I region within 9.91 kb *Pst* I -segment. DNA sequences homologous with the cloned CP gene were searched in *Bt* plasmids and chromosomal DNA by hybridization analysis (Fig. 4). The 1.15 kb *Hind*III-*Hind*III segment in the insert of pHP206 (see Fig. 3) is undoubtedly located within the coding region of the CP gene because of the gene size of about 3.5 kb. This fragment was cloned into pUC119 vector for purification, and the recombinat plasmid was used as a probe after labeling with digoxigenin. This probe hybridized with the 51.1 kb plasmid (pA) as well as with the 21.1 kb *BamH* I -and the 9.91 kb *Pst* I -fragments from *Bt* plasmids but not with chromosomal DNA (Fig. 4B). When pT509ΔP7 containing the CP gene and its 5′- and 3′- flanking regions (9.91 kb *Pst* I -segment) was used as a probe (Fig. 4C), the 19.3 kb plasmid (pB), the 11.4 and 18.5 kb *BamH* I -fragments, and the 2.15 and 4.15 kb *Pst* I -fragments were detected in addition to the bands detected with the CP gene probe. These results indicate that the cloned gene is derived from the largest plasmid and the only CP gene in this BT strain. The existence of some repeative se-

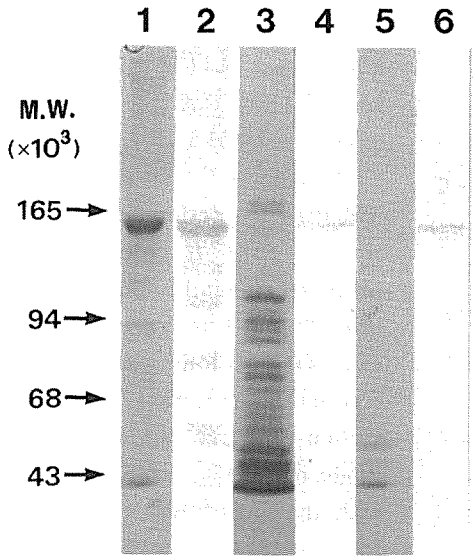

Fig. 2. SDS-polyacrylamide gel electrophoretic analysis of proteins produced by *E. coli* transformant T509.
Coomassie Brilliant Blue-stained gel (lanes 1, 3 and 5) and immunoblot with anti-crystal protein antibodies (lanes 2, 4 and 6). Lanes: 1 and 2, the crystal protein; 3 and 4, "T509 sup fraction"; 5 and 6, "T509 ppt fraction".

quences was suggested in the regions neiboring the CP gene and the smaller plasmid (pB).

DISCUSSION

B. thuringiensis dendrolimus T84A1 used in the present study carries three plasmids. The fact that a strain harbors multiple plasmids is not unusual but rather common among the various *Bt* strains (KRONSTAD *et al.*, 1983) except the *sotto* strain (SHIBANO *et al.* 1986). The cloned gene is derived from the largest plasmid, and no additional CP gene was detected. Several strains have multiple genes, and its remarkable examples is the *entomocidus* 60.5 strain which bear five lepidopteran toxin genes (VISSER *et al.*, 1988). The restriction enzyme map of the cloned DNA segment from the *dendrolimus* strain was compared with them of the *kurstaki* and *sotto* strains (Fig. 5). The sequence of the coding region showed a good correlation with them of the two other strains. To date, we determined partial nucleotide sequences which are

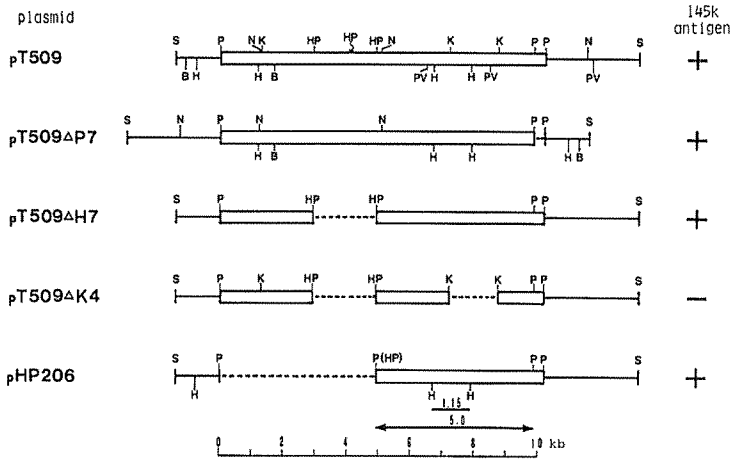

Fig. 3. Restriction enzyme map of pT509 and its deletion derivative and 145 k antigen-productivity of cell harboring recombinant plasmids. Open boxes, dashed lines and solid lines represent the remaining portion of *B. thuringiensis* DNA, deleted portion and pBR322 vector DNA, respectively. In the right column, the results of enzyme immunoassay are shown by a plus (positive) or minus (negative) sign. The arrow indicates the portion on which the CP gene resides. Abbriviation: S, *Sal* I ; B, *BamH* I ; H, *Hind*III; P, *Pst* I ; N, *Nde* I ; K, *Kpn* I ; HP, *Hpa* I ; PV, *Pvu* II.

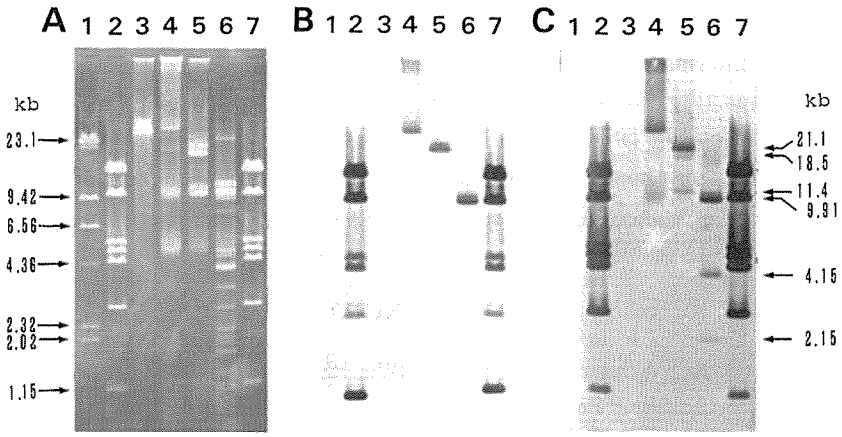

Fig. 4. Hybridization analysis of *B. thrigiensis* plasmids, their fragments and chromosomal DNA.
(A) Ethidium bromide-stained gel. (B) Southern blot hybridized with the crystal protein gene probe, pUC119 inserted by the 1.15 kb *Hind*III-segment within the crystal protein gene (see Fig. 3). Lanes: 1, λDNA-*Hind*III digest; 2 and 7, pT509ΔP7-*Sal* I +-*Pst* I +-*Hind*III; 3, *Bt* total cell DNA; 4, *Bt* plasmids; 5, *Bt* plasmids-*BamH* I ; 6, *Bt* plasmids-*Pst* I .

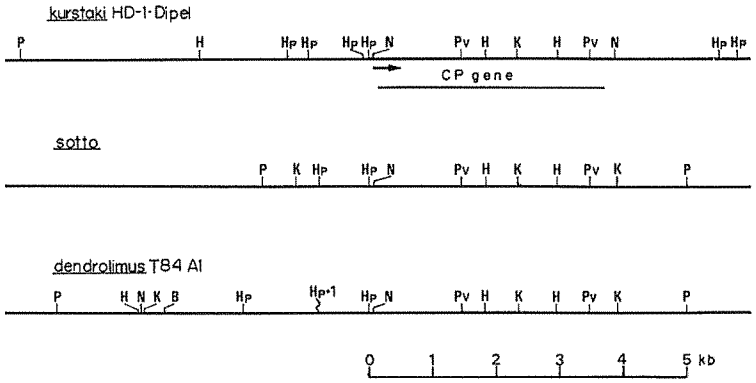

Fig. 5. Comparison between restriction enzyme maps of the crystal protein genes and the adjacent regions from *B. thuringiensis kurstaki* HD-1, *sotto* and *dendrolimus* T84A1 strains.
The arrow indicates the orientation of the crystal protein gene. Abbriviations: P, *Pst* I ; H, *Hind*III; N, *Nde* I ; K, *Kpn* I ; B, *BamH* I ; Hp, *Hpa* I ; Pv, *Pvu* II . The location of *Hpa* I ·1 site is uncertain, giving 0.83 and 1.14 kb *Hpa* I -fragments.

located in several portions over the coding region and account for about 60% of an overall number of nucleotides, and found them to be exactly the same as the sequence of the *sotto* CP gene so far (Ogoh and Nagamatsu, unpublished data). These results suggested that the crystal protein from the *dendrolimus* T84A1 strain was classified into type A (Höfte *et al.*, 1988). This prediction is well consistent with the fact that this crystal is highly toxic to *Bombyx mori* but has no detectable activity against *Spodoptera littoralis*. The mechanism of specificity of insecticidal proteins is a very interesting subject from either scientific or ap-

plied points of view. Some researchers are trying to explain the presence of multiple genes by capability of intermolecular movements of CP genes. Comparison between the restriction enzyme maps (Fig. 5) revealed structral differences of 5′-regions neiboring the CP genes. From *Bt* plasmid DNAs, Mahillon *et al.* (1987) found several repetitive sequences which are essential for transposon structures. Hybridization analysis in the present study also suggested the existence of some repetitive sequences around the CP gene and in the smaller plasmid.

REFERENCES

Birnboim, H. D., 1983, In "Methods in Enzymology" (Wu, R., Grossman, L. and Moldava, K. ed.), Vol. 100, pp. 243-255, Academic Press, New York.

Ge, A. Z., Shivarova, N. I. and Dean, D. H., 1989, Location of the *Bombyx mori* specificity domain on a *Bacillus thuringiensis* δ-endotoxin protein. *Proc. Nathl Acad. Sci. USA,* 86:4037-4041.

Höfte, H., van Rie, J., Jansens, S., van Houtven, A., Vanderbruggen, H. and Vaeck, M., 1988, Monoclonal antibody analysis and insecticidal spectrum of three types of lepidopteran-specific insecticidal crystal proteins of *Bacillus thuringiesis. Appl. Environ. Microbiol.,* 54:2010-2017.

Kondo, S., Tamura, N., Kunitake, A., Hattori, M., Akashi, A. and Ohmori, I., 1987, Cloning and nucleotide sequencing of two insecticidal δ-endotoxin genes from *Bacillus thuringiensis* var. *kurstaki* HD-1 DNA. *Agric. Biol. Chem.,* 51:455-463.

Kronstad, J. W., Schnepf, H. E. and Whiteley, H. R., 1983, Diversity of location for *Bacillus thringiensis* crystal protein genes. *J. Bacteriol.,* 154:419-428.

Mahillon, J., Seurinck, J., Delcour, J. and Zabeau, M., 1987, Cloning and nucleotide sequence of different iso-IS231 elements and their structural association with Tn4430 transposon in *Bacillus thuringiensis. Gene,* 51:187-196.

Maniatis, T., Frisch, E. F. and Sambrook, J., 1982, "Molecular Cloning", Cold Spring Harbor Laboratory, New York.

Nagamatsu, Y., Itai, Y., Hatanaka, C., Funatsu, G. and Hayashi, K., 1984, A toxic fragment from the entomocidal crystal protein of *Bacillus thuringiensis. Agric. Biol. Chem.,* 43:611-619.

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

Schnepf, H. E., Wong, H. C. and Whiteley, H. R, 1985, Amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. *J. Biol. Chem.,* 260:6264-6272.

Schnepf, H. E. and Whiteley, H. R., 1985, Delineation of a toxin-encoding segment of a *Bacillus thuringiensis* crystal protein gene. *J. Biol. Chem.,* 260:6273-6280.

Shibano, Y., Yamagata, A., Amachi, T., and Takanami, M., 1986, In "Bacillus Molecular Genetics and Biotechnology Applications" (Ganesan, A. T. and Hoch, J. H. ed.), pp. 307-320, Academic Press, New York.

Shimizu, M., Oshie, K., Nakamura, K., Takada, Y., Oeda, K. and Ohkawa, H., 1988, Cloning and expression in *Escherichia coli* of the 135-kDa insecticidal Protein Gene from *Bacillus thuringiensis* subsp. *aizawai* IPL7. *Agric. Biol. Chem.,* 52:1565-1573.

Southern, E. M., 1975, Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.,* 98:503.

Visser, B., van der Salm, T., van den Brink, W. and Folkers, G., 1988, Genes from *Bacillus thuringiensis entomocidus* 60.5 coding for insect-specific crystal proteins. *Mol. Gen. Genet.,* 212:219-224.

Bacillus thuringiensis dendrolimus T84A1 の殺中性蛋白質遺伝子のクローニング

柴田 次郎・田宮 理克・永松 康徳

広島大学生物生産学部
東広島市 724

B. thuringiensis (*Bt*) *dendrolimus* T84A1 のプラスミドから，殺虫性結晶蛋白質の遺伝子を大腸菌によってクローン化した。

本菌は10.0, 19.3と51.1 kb の3種のプラスミドを保有していた。*Pst* I 消化で生じた断片をベクター pBR322 と連結して大腸菌を形質転換した。結晶蛋白質の抗体と反応し同じ分子量の蛋白質を生産する1株が得られた。これは 10.22 kb の *Bt* DNA を含む組換えプラスミドをもち，欠失実験と毒素生産能分析から 5 kb の *Hpa* I -*Pst* I に領域に遺伝子が認められた。ハイブリダイゼイション分析から，クローン化された遺伝子が最も大きいプラスミドに由来すること，本菌中唯一のものでること，その周辺に何らかの反復配列があることが明らかになった。本遺伝子部は *sotto* 株のそれと類似していたが，5′-隣接部の変化が認められた。