Plasmids and Insecticidal Activity of Delta-endotoxin Crystals from *Bacillus thuringiensis* var. *israelensis*

Michio HIMENO, Masao IKEDA, Kikuo SEN, Naoto KOYAMA,
Tohru KOMANO, Haruyasu YAMAMOTO*
and Isamu NAKAYAMA*

Laboratory of Biochemistry, Department of Agricultural Chemistry,
Kyoto University, Kyoto 606, Japan

*Sumitomo Chemical Co., Ltd., Takarazuka Research Center,
Takatsukasa 4-chome, Takarazuka, Hyogo 665, Japan

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The insecticidal activities of 12 strains of *Bacillus thuringiensis* var. *israelensis* and purified delta-endotoxin crystals from the 4Q1 strain against mosquito larvae, *Culex pipiens pallens*, were measured and then compared with those of *B. thuringiensis* var. *kurstaki* and *Bacillus sphaericus*. Plasmid DNAs in acrySTALLIFEROUS or crystalliferous strains of those bacteria and varieties were isolated and analyzed by agarose gel electrophoresis. There were at least 7 plasmids in the 4Q1 strain.

To determine the specific plasmid coding the mosquito-toxic endotoxin protein, the relationship between the activities and the plasmid patterns of those strains was investigated. The strains harboring the plasmid pBTI-6 NDA (70 megadaltons) had insecticidal activity even though they were acrySTALLIFEROUS mutants, but all strains lacking the plasmid pBTI-6 DNA did not have the activity.

Bacillus thuringiensis var. *israelensis* (sero-type H14) is known as a bacterial pathogen against mosquito¹⁾ and black fly²⁾ larvae. A parasporal body is called a proteinous crystalline inclusion, delta-endotoxin crystal, or simply the crystal, which is produced in the sporulating cells of *B. thuringiensis* and this crystal is only composed of the toxin protein.³⁾ A few cultures of *B. thuringiensis* are used as commercial insecticidal formulations, because the crystals of *B. thuringiensis* var. *kurstaki* and several other varieties are toxic to many lepidopteran insects and that of *B. thuringiensis* var. *israelensis*^{4,5)} is toxic to dipteran insects. It was reported that the molecular weight of lepidopteran toxic protein was about 68,000 but that of dipteran toxic protein was 26,000. The antiserum against the lepidopteran toxic protein did not react with the dipteran toxin and also the antiserum against dipteran toxic protein did not react with the lepidopteran toxin.³⁾

Recently, it was suggested that the delta-endotoxin protein gene might be encoded in a specific plasmid as shown by curing experiments,^{6,7)} when the lepidopterous toxic strains were used, in which the loss of the crystal formation was associated with the loss of the specific plasmid. When a certain plasmid was transferred into an acrySTALLIFEROUS strain of *B. thuringiensis* or *Bacillus cereus*, these strains were transformed into crystal-forming strains.⁸⁾ The delta-endotoxin protein genes from four strains, *B. thuringiensis* var. *kurstaki* HD1-Dipel,⁹⁾ *kurstaki*,¹⁰⁾ *berliner* 1715¹¹⁾ and *kurstaki* HD73,¹²⁾ carrying strong insecticidal activity against lepidopterous insects, were cloned in a strain of *Escherichia coli*. The homologous region to the cloned delta-endotoxin protein gene¹³⁾ was distributed on plasmids or on chromosomal DNA in various varieties except *israelensis*, *kyushuensis*, *indiana*, and *dakota*. The nucleotide sequence of the promoter region and part of the

coding region of the delta-endotoxin protein gene cloned from the plasmids in the HD1-Dipel strain were determined¹⁴⁾ and the sequence of the promoter region of the crystal protein gene cloned from chromosomal DNA of the *berliner* 1715 was also determined.¹⁵⁾

On the other hand, it was suggested that the delta-endotoxin protein gene of the *israelensis* strain was encoded on a high molecular weight plasmid of a indeterminate size (Kamdar and Jayaraman¹⁶⁾), on a 72 megadaltons (Md) plasmid in 9 plasmids (Ward and Ellar¹⁷⁾), and on a small plasmid (4.0~4.4 Md) in 3 plasmids (Faust *et al.*¹⁸⁾). However, the relationship between the insecticidal activities and the plasmid patterns of those strains was not clear.

In this paper, the plasmids in 12 strains of *israelensis*, the derivatives from the 4Q1 strain, 2 strains of *kurstaki*, and 3 strains of *B. sphaericus* were isolated and were analyzed by agarose gel electrophoresis. The insecticidal activities of these strains were measured against mosquito larvae. It is concluded that a 70 Md plasmid in the *israelensis* strains is associated with the ability to produce the delta-endotoxin protein.

MATERIALS AND METHODS

Strains and mediums. Table I shows bacterial species, strains, varieties, and sources of bacteria used in this study. *Bacillus thuringiensis* and *Bacillus sphaericus* were cultured in PY broth medium¹⁹⁾ (polypeptone 10 g, yeast extract 5 g, NaCl 5 g per liter, pH 7.0) or GNB broth medium²⁰⁾ [polypeptone 10 g, beef extract (dry powder) 5 g, glucose 1 g, NaCl 2 g per liter, pH 7.0] at 30°C. *B. subtilis* was grown in Pennassay broth medium (Difco Lab.) or PY broth medium at 37°C for isolation of standard plasmid DNA. *Escherichia coli* was cultured in L broth medium at 37°C overnight. The *Bacillus* strains were grown on a GNB agar plate composed of GNB broth medium containing 1.5% agar (GNB agar medium) for 7~8 days at 30°C, and then formation of the delta-endotoxin crystal in the *Bacillus* strains was observed with phase-contrast microscopy.

The *israelensis* strains [4Q1 and ONR60A (IPL)] were treated with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) (0.005~0.01%) by the procedures described Nishiitsutsuji-Uwo *et al.*,²⁰⁾ and the cells were cultured on GNB agar plates containing 50 µg/ml of rifampicin. Subsequently asporogenic mutants in the rifampicin-

resistant cells were selected by the method described Nishiitsutsuji-Uwo *et al.*²⁰⁾

Purification of delta-endotoxin crystals. An inoculum of *B. thuringiensis* var. *israelensis* 4Q1 strain was spread on 4 GNB agar plates (35×35 cm) by a glass spreader and incubated at 28°C for 7 days. Sporangia containing spores and the endotoxin crystals were disrupted with an ultrasonic oscillator (Ultrasonic Generator 4280, Kaijo Denki Co., Ltd.) for 10-min intervals in an ice-bath. The disrupted cells were suspended in water and centrifuged at 15,000×*g* for 15 min at 4°C. The delta-endotoxin crystals in the pellets were separated and purified by the biphasic separation technique described by Goodman *et al.*,²¹⁾ and this procedure was repeated 8~10 times. The delta-endotoxin crystals were washed with distilled water several times and centrifuged at 15,000×*g* for 15 min, and finally 85 mg of purified dry crystals were obtained after lyophilization.

Preparation of crude sample to determine the insecticidal activity against mosquito larvae. *B. thuringiensis* strains for assay of the insecticidal activity against mosquito larvae were grown in 100 ml of GNB broth medium at 28°C for 7~8 days with vigorous shaking. The cells were centrifuged at 12,000×*g* for 30 min and then suspended in 10 ml of distilled water. The suspensions were frozen in dry ice-ethanol and thawed three times and subsequently homogenized with an ultrasonic oscillator (Handy Sonic, model UR-20p, Tomy Seiko Co., Ltd.) for 5 min at 1-min intervals in an ice-bath. The disrupted cell suspensions were centrifuged at 12,000×*g* for 30 min at 4°C. The pellets were suspended in 3 ml of distilled water. Just before use for assay of LC₅₀ the pellets were homogenized with the ultrasonic oscillator again. Asporogenic mutants of the 4Q1 strain and the *B. sphaericus* strains were cultured in 100 ml of GNB broth medium as described above. After harvesting the cells, they were suspended in 3 ml of distilled water and subsequently disrupted by freezing and thawing, and homogenized with the ultrasonic oscillator. The suspensions of disrupted cells were directly used for the assay of LC₅₀ after homogenization with the oscillator. The dry weights of the usual crude sample extracted from the 4Q1 strain and the asporogenic mutants were 13.3~18.7 mg/ml.

Assay of insecticidal activity. Mosquito larvae, *Culex pipiens pallens* Coquillett, used in this experiment were reared in this laboratory (Sumitomo Chemical Co., Ltd.) with an artificial diet containing a mixture (1:1) of finely ground rabbit chow and dry brewers' yeast at 27°C. The insecticidal activities were measured in duplicate and 20 third-instar larvae were placed in a cup containing 100 ml of distilled water adding the serial dilution of the purified delta-endotoxin crystals or crude samples. The cups holding larvae were maintained with a photoperiod of 16 hr of light and 8 hr of darkness at 25°C for 3 days. Mortalities were counted at 24 hr or/and 48 hr and lethal concentration (LC₅₀) values were expressed as ppm (w/w) re-

TABLE I. BACTERIAL STRAINS USED IN DETERMINATION OF THE INSECTICIDAL ACTIVITY AND/OR ISOLATION OF PLASMID DNA

Species	Strain name ^a	Source and reference
<i>Bacillus thuringiensis</i> var. <i>israelensis</i>	HD500	USDA ^b and ARM ^c
	HD500A	This work
	HD500B	This work
	HD522	USDA and ARM
	HD563	USDA and ARM
	HD567	USDA and ARM
	HD648	USDA and ARM
	HD654	USDA and ARM
	HD756	USDA and ARM
	4Q1	BGSC and (ONR60A) ^d in BGSC catalog ^e
	4Q4	BGSC and (HD657) ^d in BGSC catalog
	ONR60A (Bulla)	T. Iizuka ^f
	ONR60A (IPL)	T. Iizuka
	4Q1-54	This work
<i>Bacillus thuringiensis</i> var. <i>kurstaki</i>	HD1	USDA and ARM
	4D1	BGSC and (HD1) ^d in BGSC catalog
<i>Bacillus thuringiensis</i> var. <i>thuringiensis</i>	HD2	USDA and ARM
<i>Bacillus sphaericus</i>	13A1	USDA and (1593) ^d in BGSC catalog
	2297	Y. Izumi ^g
	SSII-1	Y. Izumi
<i>Bacillus subtilis</i> 168	1E6	BGSC and BGSC catalog, harboring pUB110
	1E21	BGSC and BGSC catalog, harboring pBD8
	1E24	BGSC and BGSC catalog, harboring pBD10
	1E25	BGSC and BGSC catalog, harboring pBD11
	1E27	BGSC and BGSC catalog, harboring pBD9
<i>Escherichia coli</i>	C600	T. Uozumi, ^h harboring pTA1302
	459	W. Goebel, ⁱ harboring pJK3

^a Strain name was shown by HD number (Dulmage culture number described in ARM), BGSC number described in BGSC catalog or original name.

^b Provided by Dr. T. Yamamoto, Cotton Insect Research, ARS, USDA, U.S.A.

^c ARM; US Department of Agriculture Research Service. Agricultural Reviews and Manuals. ARM-S-30/October, 1982.

^d (ONR60A), (HD657), (HD1) and (1593) are written as original code in the catalog.

^e BGSC; The *Bacillus* Genetic Stock Center, Catalog of Strains, The Ohio Univ., U.S.A.

^f Provided by Dr. T. Iizuka, Hokkaido Univ., Japan.

^g Provided by Dr. Y. Izumi, Kyoto Univ., Japan, who originally obtained it from Dr. E. W. Davidson,³⁰⁾ Arizona State Univ., U.S.A.

^h Provided by Dr. T. Uozumi, the Univ. of Tokyo, Japan.

ⁱ Provided by Dr. W. Goebel, Univ. Würzburg, West Germany.

quired to cause 50% mortality of the larvae. An average of the two experiments is shown in Table II.

Isolation of plasmid DNA. The basic procedure used for extraction of small plasmid DNA (<10 Md) followed the procedure described by Davis *et al.*,²²⁾ and for large plasmids followed the procedure described by Birnboim and Doly.²³⁾ Plasmids pUB110 (3.0 Md),²⁴⁾ pBD8 (6.0 Md), pBD9 (5.4 Md), pBD10 (4.4 Md), pBD11

(4.0 Md), pTA1302 (5.2 Md), and pJK3 (7.6 Md)²⁵⁾ were isolated from *B. subtilis* 168 strains (1E6, 1E21, 1E27, 1E24, and 1E25), *E. coli* C600, and *E. coli* 459, respectively. Total plasmid DNAs of *B. thuringiensis* HD1 and HD2 strains were also used as plasmid DNA markers.⁷⁾

Agarose gel electrophoresis. Agarose gel electrophoresis was performed in Dotite agarose II (Dojindo Lab.,

Kumamoto, Japan) at a concentration of 0.4~0.7% containing ethidium bromide (EtBr) (1 µg/ml) using a horizontal apparatus (15 × 20 × 0.4 cm³). Electrophoretic migration was done out at 40 V for 1 hr and then at 100 V for 4 hr in Tris-acetate buffer (2 mM EDTA, 40 mM Tris, 20 mM CH₃COONa, pH 7.4) containing EtBr, one quarter volume of the dye mixture (0.1% bromophenol blue, 50% sucrose, 1 mM EDTA) was added to the plasmid sample prior to loading and 15~25 µl of the sample was applied into a well. Plasmid DNA was visualised by illumination with a UV lamp (λ=2536Å) and photographed with a Mamiya RB67 camera (Polaroid type 665 positive/negative film) using a red filter.

Two-dimensional gel electrophoresis for determination of covalently closed circular (ccc), open circular (oc), or

linear (l) DNA in plasmid DNA samples was by a modified technique of Hintermann *et al.*²⁶⁾ Sample {40 µl of DNA sample [4Q1 or ONR60A (IPL)]} were first run on 0.5% agarose gel in one direction at 40 V for 30 min, and then at 3 V/cm for 4 hr in the Tris-acetate buffer containing EtBr. The gel was exposed to UV light (15 W Toshiba germicidal lamp at a height of 30 cm). The agarose gel was then turned 90° and run as described above. Before detection of plasmid bands the gel was then soaked in the electrophoresis buffer containing 2 µg/ml of EtBr for 30 min.

Reagents and enzymes. Yeast extract, Pennassay broth, and Bacto-tryptone were purchased from Difco Lab. Lysozyme, RNase A, and SDS were from Sigma Chemical

TABLE II. LC₅₀ OF 13 STRAINS OF *Bacillus thuringiensis* AND 3 STRAINS OF *Bacillus sphaericus* TO MOSQUITO LARVAE

Species	Strain	Exp. ^a	LC ₅₀ (ppm) ^b	
			At 24 hr	At 48 hr
<i>B. thuringiensis</i> var. <i>israelensis</i>	4Q1 ^d (purified)	1	0.0060 (0.0032~0.010) ^c	0.0050 (0.0028~0.0093)
	4Q1	1	2.0 (1.1~3.7)	1.6 (1.0~3.4)
	4Q1	2	2.1 (1.7~2.7)	1.3 (1.1~1.6)
	HD500	2	5.8 (2.8~9.2)	3.6 (2.2~6.1)
	HD522	2	0.31 (0.25~0.37)	0.25 (0.19~0.32)
	HD563	2	1.0 (0.87~1.1)	0.68 (0.47~1.2)
	HD567	2	> 100	> 100
	HD648	2	9.4 (7.2~13)	4.5 (3.4~5.9)
	HD654	2	0.50 (0.43~0.60)	0.47 (0.40~0.56)
	HD756	2	> 100	> 100
	ONR60A (Bulla)	2	12 (9.4~15)	7.4 (6.0~9.0)
	ONR60A (IPL)	2	> 100	> 100
	ONR60A (IPL)	3		> 1,000
	4Q4	3		> 1,000
	4Q1	4		0.21 (0.07~0.59)
	4Q1-54 ^e	4		≐ 60
<i>B. thuringiensis</i> var. <i>kurstaki</i>	HD1	2	> 100	> 100
	4D1	4		≐ 400
<i>B. sphaericus</i>	13A1	2	10~100	10~100
	2297	2	> 100	10~100
	SSII-1	2	> 100	> 100

^a Experimental number.

^b LC₅₀; lethal concentration (50% endpoint) is shown by ppm (w/w). Twenty larvae (3rd instar) of *Culex pipiens pallens* were bred in 100 ml of water at 25°C for 3 days and the average of dual experiments at 24 hr or/and 48 hr are shown in the table. The detail procedures for determination were described in MATERIALS AND METHODS.

^c Numbers within parentheses are 95% confidence limit.

^d 4Q1 (purified) shows purified 4Q1 crystals. *B. thuringiensis* 4Q1 was cultured on the GNB plates described in MATERIALS AND METHODS. The crystals were purified by the biphasic separation technique. Lyophilized crystals were suspended in water and used.

^e Sporeless and rifampicin-resistant (50 µg/ml) mutant of 4Q1, which was treated *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.005%) for selection of mutants lacking in ability to form a spore, did not produce the delta-endotoxin crystals during 8 days at 28°C.

Co. Polyethylene glycol 6,000, dextran sulfate 500, CsCl, EtBr, tris(hydroxymethyl)aminomethane, and other chemicals were bought from Nakarai Chemicals, Ltd.

RESULTS

Insecticidal activity of purified 4Q1 crystals and its crude sample

The LC_{50} value of purified delta-endotoxin crystals isolated from *B. thuringiensis* var. *israelensis* 4Q1 strain was determined with mosquito larvae and the results are shown in Table II (Exp. 1). The insecticidal activity of the purified crystals was 0.006 ppm at 24 hr. This LC_{50} value of the crystals corresponded to 6 ng/ml at 24 hr and this is a very much lower value than that described by Kamadar and Jayaraman¹⁶⁾ but higher than that by Tyrell *et al.*⁴⁾ On the other hand, in the same experiment the LC_{50} of the crude sample was 2.0 ppm.

Insecticidal activities of crude samples from various strains

To determine the insecticidal activity against mosquito larvae, crude samples were prepared from 100 ml cultures and the LC_{50} values were determined. These results are also shown in Table II (Exp. 2, 3, and 4). The order of their activities is as follows; HD522 > HD654 > HD563 > 4Q1 > HD500 > HD648 > ONR60A (Bulla) > HD567, HD756, ONR60A (IPL). ONR60A (IPL), HD567, and HD756 did not have any insecticidal activity against mosquitoes. Yamamoto and McLaughlin,²⁷⁾ and other investigators²⁸⁾ showed that the HD1 strain had activity against mosquito larvae but the LC_{50} value of the HD1 strain which was described as 4D1 strain in the catalog of strains of *Bacillus* Genetic Stock Center was 400 ppm at 48 hr. This value is much higher than that of other active strains, for example, 4Q1 or HD522. Several investigators showed that *B. sphaericus*³⁰⁾ strains also had insecticidal activities against mosquito larvae. Because the LC_{50} values of the three strains of *B. sphaericus* were 10~100 ppm or over 100 ppm, the insecticidal activity of *B. sphaericus* against mosquito larvae was

higher than that of the 4D1 strain. Ten asporogenic mutants were obtained from about 300 rifampicin-resistant mutants derived from the 4Q1 strain with mutagen (NTG) treatment, and all of these mutants have lost both the delta-endotoxin crystal- and spore-forming abilities. However, the LC_{50} value of an asporogenic mutant 4Q1-54 was about 60 ppm and this value is lower than that of ONR60A (IPL), 4Q4, HD1 and the *B. sphaericus* strains. The LC_{50} values of other mutants which lost both the crystal- and spore-forming abilities were 190~480 ppm (data not shown in Table II) and almost the same as that of 4D1 strain.

Agarose gel electrophoretic pattern of B. thuringiensis var. israelensis 4Q1 strain

Agarose gel electrophoresis of plasmid

FIG. 1. Plasmid Patterns of *B. thuringiensis* var. *israelensis* (4Q1 Strain).

The 4Q1 cells in PY broth medium were harvested at various times after inoculation and the plasmid DNAs prepared by a modification of the procedure described by Birnboim and Doly²³⁾ were electrophoresed on 0.5% agarose horizontal slab gel at 40 V for 1 hr, then at 100 V for 4 hr. The configuration of plasmids was determined by two-dimensional electrophoresis by the procedure described in MATERIALS AND METHODS. The molecular weights of the previously characterized ccc plasmids from HD1, HD2, and several bacteria harboring marker plasmids are listed on the left margin of the photograph as size references. Chromosomal DNA, ccc, oc, and linear plasmid DNAs are indicated by chr, c, o, and l, respectively, on the right margin. The numbers (1~7) beside c or o indicate pBTI-1, pBTI-2, pBTI-3, pBTI-4, pBTI-5, pBTI-6 and pBTI-7, respectively. The numbers above gel lane show the time (hr) after inoculation.

TABLE III. NUMBER AND SIZE OF PLASMID DNA DETECTED IN CRYSTALLIFEROUS AND ACRYSTALLIFEROUS STRAINS OF *B. thuringiensis* AND *B. sphaericus*

Species	Strain	Crystal	No. of plasmids	Plasmid DNA (Megadaltons)						
				3.6 pBTI-1	4.3 pBTI-2	4.9 pBTI-3	10.0 pBTI-4	63 pBTI-5	70 pBTI-6	82 pBTI-7
<i>B. thuringiensis</i> var. <i>israelensis</i>	HD500A ^a	+	6	+	+	—	+	+	+	+
	HD500B	—	5	+	+	—	+	+	—	+
	HD522	+	5	—	+	+	—	+	+	+
	HD563	+	7	+	+	+	+	+	+	+
	HD567	—	6	+	+	+	+	+	—	+
	HD648	+	7	+	+	+	+	+	+	+
	HD654	+	7	+	+	+	+	+	+	+
	HD756	—	6	+	+	+	+	+	—	+
	4Q1	+	7	+	+	+	+	+	+	+
	4Q1-54 ^b	—	5	—	+	+	+	+	+	—
	4Q4	—	4	+	+	—	—	+	—	+
	ONR60A (Bulla)	+	5	+	+	—	—	+	+	+
	ONR60A (IPL)	—	5	+	+	—	+	+	—	+
<i>B. thuringiensis</i> var. <i>kurstaki</i>	HD1	+	10	1.4, 4.9, 5.2, 5.4, 9.3, 29, 44, 52, (110), ^c (120)						
<i>B. thuringiensis</i> var. <i>thuringiensis</i>	HD2	ND ^d	9	5.2, 6.2, 7.2, 32, 37, 54, 57, 75, (150)						
<i>B. sphaericus</i>	13A1	ND	ND							
	2297	+	3	2.4, 3.4, 80						
	SSII-1	ND	ND							

^a HD500A and HD500B were isolated from HD500 and shown in Fig. 2.

^b 4Q1-54, a sporeless and rifampicin-resistant mutant, did not produce the crystals.

^c Molecular weight of plasmid DNA within parentheses was not confirmed on agarose gel electrophoresis.

^d ND; not determined.

DNA from the 4Q1 strain showed that the strain contained a number of distinct DNA bands (Fig. 1). The configuration of DNA was determined by the two-dimensional gel electrophoresis of 0.4% or 0.5% agarose. Relative molecular weights of the plasmid DNAs were determined from a standard curve of authentic plasmids in 0.4%, 0.5%, and 0.7% agarose gels. The 4Q1 strain has at least 7 distinct plasmid DNAs which were called pBTI-1, pBTI-2, pBTI-3, pBTI-4, pBTI-5, pBTI-6, and pBTI-7 in this report, which are 3.6 Md, 4.3 Md, 4.9 Md, 10.0 Md, 63 Md, 70 Md, and 82 Md, respectively (Table III). The plasmid DNAs were isolated at various times after inoculation and then the large plasmids (pBTI-5, pBTI-6,

and pBTI-7) were easily detected at 20~25 hr after inoculation (Fig. 1).

Plasmid patterns of various strains of B. thuringiensis and B. sphaericus

The plasmid DNA was analyzed by 0.5% agarose gel electrophoresis and the results are shown in Fig. 2. The number of plasmid bands detected in 4 strains (4Q1, HD563, HD648, and HD654) was 7. The HD500 strain used for the determination of the LC₅₀ value was separated into HD500A and HD500B. The HD500A (crystalliferous) strain did not contain pBTI-3, and the HD500B (acrySTALLIFEROUS) strain did not contain either pBTI-3 or pBTI-6. The 4Q1-54 strain did not have two

FIG. 2. Plasmid Patterns of Various Strains of *B. thuringiensis* and *B. sphaericus*.

The strains of *B. thuringiensis* var. *israelensis* HD500A (A), HD500B (B), HD522 (C), HD563 (D), HD567 (E), HD654 (F), HD648 (G), HD756 (H), 4Q1 (I), 4Q1-54 (J), 4Q4 (K), ONR60A (Bulla) (L), ONR60A (IPL) (M), *B. sphaericus* 1593 (N), *B. sphaericus* 2297 (O), *B. sphaericus* SSII-1 (P), *B. thuringiensis* var. *kurstaki* HD1 (Q) and *B. thuringiensis* var. *thuringiensis* HD2 (R) were cultured in PY broth medium at 30°C with shaking for 24 hr. Plasmid samples (20 μ l) were applied to slots on a 0.5% agarose slab gel and the electrophoresis was done by the procedures described in MATERIALS AND METHODS. The plasmids of HD1 and HD2 were shown as markers of molecular weight.

plasmids, pBTI-1 and pBTI-7, but contained pBTI-6. The 4Q1-54 strain was a rifampicin-resistant mutant which lost the abilities of the delta-endotoxin crystal- and the spore-formation but had the insecticidal activity. All mutants derived from 4Q1, which lost the crystal-forming ability but had the plasmid pBTI-6, showed lower insecticidal activity than that of 4Q1, but a similar activity as *kurstaki* or *B. sphaericus* strains. The *B. sphaericus* strains, HD1, and HD2 did not contain the same plasmid as that in the *israelensis* strains. All *israelensis* strains lacking the plasmid pBTI-6 did not have the insecticidal activity. These results are listed in Table III. These results suggested that the insecticidal activity of the *israelensis* strains is associated with the plasmid pBTI-6 DNA.

DISCUSSION

The insecticidal activities of the purified crystal and crude samples of *israelensis* against mosquito larvae were measured. The activity of the purified crystals from the 4Q1 strain was about 330 times higher than that of a crude sample from the same strain, indicating that

the insecticidal substance was about 0.3% of the crude sample. Also the amount of active substance was calculated as about 20% of dry matter in the crude sample. It is apparent that the active substance in dry matter is almost crystalline protein but not any other toxic substance like beta-exotoxin, because water insoluble materials were collected from the cell lysate. The plasmid patterns obtained from *israelensis* were shown by Iizuka *et al.*²⁹⁾ but they did not discuss the correlation between a specific plasmid and the toxicity. Faust *et al.*¹⁸⁾ suggested that loss of a specific plasmid (4.0 ~ 4.4 Md) was associated with loss of the crystal production, while it was proposed by Kamdar and Jayaraman¹⁶⁾ that loss of both of the insecticidal activity and the crystal production was correlated to loss of a large plasmid and Ward and Ellar¹⁷⁾ showed that loss of the 72 Md plasmid was invariably accompanied with loss of the ability to synthesize the insecticidal delta-endotoxin protein.

After this manuscript was submitted, González and Carlton³¹⁾ reported the plasmid patterns of crystalliferous and acrySTALLIFEROUS mutants of the HD567 strain and concluded that a 75 Md transmissible plasmid was impli-

cated in the crystal production. However the HD567 strain used in our experiments did not contain the 70 Md (or 75 Md) plasmid, the crystal-forming activity and also the insecticidal materials. This contradiction might be due to the spontaneous loss of the 70 Md plasmid by our HD567 strain.

This investigation has been performed independently from other investigators and the relationship between the LC_{50} values and the plasmid patterns of 12 *israelensis* strains is reported. Also in this report we showed that all strains carrying the insecticidal activity always contained the 70 Md plasmid and suggested that the 70 Md plasmid (pBTI-6) was required for their insecticidal activities, even in those *israelensis* strains which lost the crystal-producing ability.

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