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Restriction Endonuclease Maps of Three Plasmids from *Bacillus thuringiensis* serovar *israelensis* 4Q1

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Bacillus thuringiensis serovar *israelensis* 4Q1로부터 분리된 plasmid 제한효소지도 작성

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Bacillus thuringiensis serovar *israelensis* 4Q1 contains 8 different covalently closed-circular (ccc) plasmids of molecular weight 204, 167, 109, 103, 16, 7.6, 6.4, and 5.0 kb. The three smallest plasmids, designated pBti6, pBti7, and pBti8 may prove to be useful as cloning vectors because of their size and ease of isolation. The three plasmids were incubated separately with 9 different restriction enzymes and 7 of the enzymes tested cleaved one or more of the plasmids. Plasmid pBti6 has a single site for *Bgl* II, *Pst* I and *Pvu* II, two sites for *Bcl* I and *Eco* RI, and five sites for *Hind* III. Plasmid pBti7 has a single site for *Bam* HI and *Pst* I, two sites for *Hind* III, and three sites for *Pvu* II. Plasmid pBti8 has a single site for *Bam* HI, *Bcl* I and *Hind* III, two sites for *Eco* RI, and three sites for *Bgl* II and *Pvu* II. Composite restriction enzyme maps for pBti6, pBti7 and pBti8 were constructed. The sites of restriction enzyme cleavage were determined by single, double and partial digests of the plasmid DNA. All the restriction sites were aligned relative to the single *Bgl* II (pBti6), *Pst* I (pBti7), or Hind III (pBti8) site, respectively.

Plasmids in the entomocidal bacterium, *Bacillus thuringiensis* have been reported by a number of investigators (Faust et al. 1979; Gonzalez et al., 1981; Iizuka et al., 1981). They are of interest in terms of their potential as cloning vectors for genetic engineering (Faust and Bulla, 1982) and because of their probable δ-endotoxin encoded functions (Faust et al., 1983; Gonzalez et

al., 1981; Gonzalez et al., 1982; Gonzalez and Carlton, 1984; Held et al., 1982; Kamdar and Jayaraman, 1983; Klier et al., 1982; Schnepf and Whiteley, 1981; Ward et al., 1984; Ward and Ellar, 1983; Wong et al., 1983). The δ-endotoxin is a protein that appears during the sporulation phase as a crystalline inclusion toxic to many lepidopteran larvae (see review of Faust and Bulla, 1982). Additionally, recent investigations have provided evidence for the existence of several *B. thuringiensis* strains (e.g., serovars *israelensis, darmstadiensis*) that possess preferential toxicity to mosquitoes and blackflies (deBarjac, 1978; Goldberg and Margalit, 1977; Ohba and Aizawa, 1979; Padua et al., 1980, 1984; Undeem and Nagel, 1978).

Several interesting possibilities for genetic engineering with *B. thuringiensis* are to broaden the host range specificity by introducing several crystal genes of different origins in the same recipient strain, to increase the toxicity producing capacity, or to develop shuttle-cloning vectors for transferring the δ-endotoxin gene into other entomopathogens having recyclibility (e.g., *B. sphaericus*) or into the genetic background of the insect's food source (e.g., algae/mosquitoes). *B. thuringiensis* serovar *israelensis* contains up to 8 different plasmids of molecular weights (M_r) ranging from 200 down to about 5 kb (Faust et al., 1983; Gonzalez and Carlton, 1982; Gonzalez and Carlton, 1984; Kamdar and Jayaraman, 1983; Lereclus et al., 1982; Ward and Ellar, 1983). Reports have implicated at least 4 different plasmids in δ-endotoxin synthesis in plasmid curing experiments: a 109 kb plasmid (Gonzalez and Carlton, 1984; Ward and Ellar, 1983), a 6.4kb plasmid (Faust et al., 1983), a plasmid of indeterminant size (Kamdar and Jayaraman, 1983) and a 7.3kb plasmid (Clark et al., 1984).

Unfortunately, the curing experiments do not distinguish between the loss of a structural gene and the loss of a plasmid-encoded regulatory gene. In this regard, Ward et al (1984) have reported the isolation of a 9.7 kb *Hind* III restriction fragment from the 109 kb plasmid that encodes the mosquitocidal δ-endotoxin gene. The fragment was inserted into the vector pUC12 and transformed in *Escherichia coli*. Assembly into the unique crystalline inclusion apparently did not occur in the host. Minnich and Aronson (1984) have demonstrated that plasmid involvement in δ-endotoxin production in *B. thuringiensis* serovar *kurstaki* appears to be conditionally expressed. A role for at least some of the cryptic plasmids found in most *B. thuringiensis* strains in regulating δ-endotoxin synthesis was supported by the uncovering of a conditional phenotype with loss of a 167 kb plasmid and the increased synthesis of δ-endotoxin in transcipients containing one or two 7.6- to 9.1 kb plasmids in addition to the larger δ-endotoxin encoding plasmids. A 44 kb plasmid also appeared to be involved in regulation in addition to containing a copy of the δ-endotoxin gene.

Because the genes for δ-endotoxin have usually been located on large plasmids (50 to 230 kb) and large plasmids generally have low copy numbers, the smaller plasmids found in *B. thuringiensis* may prove to be more useful as cloning vectors because of their size and ease of isolation and transfer. Presumably, the presence of the gene on a plasmid with the potential for a higher copy number rather than a chromosomal gene or large plasmid could contribute to an increased synthesis of this toxic protein during sporulation.

As a necessary step to developing cloning and gene engineering systems with indigenous plasmids harbored by *B. thuringiensis* and investigating their functions, we have delineated tentative physical maps of the three small plasmids, designated pBti6, pBti7 and pBti8 harbored by *B. thuringiensis* serovar *israelensis* 4Q1.

MATERIALS AND METHODS

Cell growth and isolation of plasmids

B. thuringiensis serovar *israelensis* 4Q1 was obtained from Dr. D. H. Dean, *Bacillus* Genetic Stock Center, Columbus, OH. Cells were grown for 6 h at 30°C (150rpm) in 50 ml Difco nutrient broth and then transferred to 200 ml of Spizizen's minimal phosphate medium (Spizizen, 1958) sup-

plemented with 0.5% glucose (added as a sterile 50% solution) and 0.1% Difco vitamin-free casamino acids for 2-3h ($OD_{600} \cong 0.45$). The cells were harvested by centrifugation at 1500g for 20 min and resuspended in 8 ml of TES buffer (20 mM Tris base, 5mM disodium EDTA, 50mM NaCl, pH8.0) containing 20% sucrose and 2 mg/ml egg white lysozyme-200 μg/ml RNase A (Sigma) in a polypropylene centrifuge tube. The cell suspension is vortexed vigorously and incubated at 37°C for 30 to 60 min (until spheroplasts are generated), then lysed by the addition of 4 ml 6% sodium dodecyl sulfate (SDS) in electrophoresis buffer (89 mM Tris base, 2.5mM disodium EDTA, 89mM boric acid, pH 8.3) containing 5% sucrose and mixed gently (If the suspension does not clear, a quick snap of the the wrist to mix the suspension forcibly results in clearing; this action does not seem to affect the integrity of the plasmids). An equal volume of 4 M NaCl was added to the cleared lysate, and the whole mixture was left overnight at 4°C. The mixture was centrifuged for 25min at 20,000g, the supernatant fluid was carefully decanted, any remaining white flocculent material in the supernatant fluid was removed with a Pasteur pipette, and two volumes of cold 95% ethanol were added. The mixture was stored overnight at -20°C, and then centrifuged at 20,000g for 20min; the supernatant fluid was carefully removed, any remaining ethanol evaporated under vacuum, and the plasmids resuspended in 0.5-1.0 ml Tris buffer (10 mM Tris-5 mM NaCl-1 mM EDTA, pH 7.4).

Plasmid DNAs were resolved on vertical 0.5% agarose gels (low electroendosmosis, FMC Corp.) in the Tris-borate electrophoresis buffer. The samples (200 μl) were adjusted with 20 μl of 0.25% bromophenol blue in 50% glycerol (in electrophoresis buffer.) The DNA preparations were applied to the sample slots in 10-70 μl volumes. Gels were run at constant current in three stages: 1h at 3.0 mA, then 30min at 7.0 mA, and finally 3.5 h at 28 mA (120V). The gels were stained in electrophoresis buffer with 1μg of ethidium bromide/ml for 30 min, destaned in distilled water for 4-5h, and photographed on a transilluminator (Model TM-36, Ultraviolet Products, Inc., San Gabriel, CA) with Polaroid type high-speed No. 55 film and a Kodak No. 23A red filter.

Restriction enzyme analysis

For restriction enzyme analysis, plasmids were isolated as described before except that the plasmid preparation was banded at least twice in CsCl-ethidium bromide gradients, and then electrophoresed on the agarose gels. Recovery of the plasmids from the agarose gels was accomplished by excision and electroelution as described by Maniatis et al. (1982). The restriction endonucleases *Bam* HI, *Bcl* I, *Bgl* II, *Eco* RI, *Hind* III, *Kpn* I, *Pst* I, *Pvu* II, and *Sal* I were obtained from Bethesda Research Laboratories, Inc. Analysis and construction of maps of sites cleaved by the restriction endonucleases were essentially as described by Maniatis et al. (1982). Incubations were performed in a volume of 11 μl at 37°C for at least 2 h and up to 12 h with a twofold excess of enzyme. Digestion conditions were essentially as specified by the supplier. In the case of double digests, enzymes were added sequentially with appropriate adjustment of the buffer for the second enzyme, when necessary. Before electrophoresis, samples of digested DNA were made 20mM in EDTA, 15% in sucrose and 0.1% in bromophenol blue.

Digests of the three plasmids were initially resolved on 0.7% agarose gels. However, since a number of small fragments difficult to size on agarose gels were generated, the digest products were subsequently fractionated by gel electrophoresis on a vertical discontinuous polyacrylamide slab gel, consisting of a 3% polyacrylamide gel on top of a 10% polyacrylamide gel layer in order to resolve both small and large fragments as described by van den Hondel and Schoenmakers (1975). Marker fragments of *Hind* III digests of λ DNA and *Hae* III digests of ϕ X174 DNA were used as M_r standards.

RESULTS AND DISCUSSION

Isolation of plasmids

As seen in Fig. 1 strain 4Q1 of *B. thuringienis* serovar *israelensis* contains nine extrachromoso-

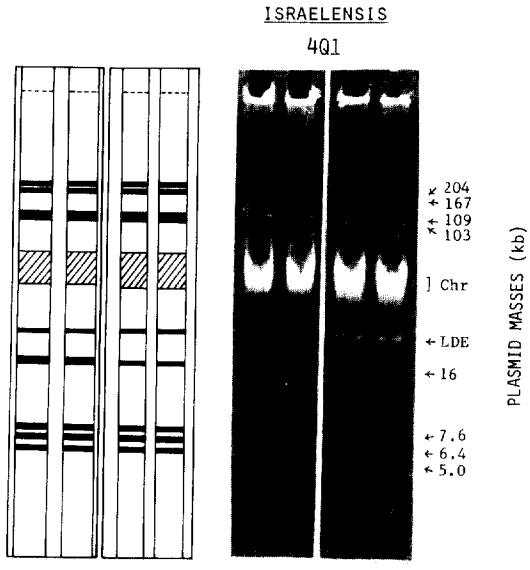

Fig. 1. *Typical electrophoretic pattern of plasmid DNA for Bacillus thuringiensis serovar israelensis 4Q1 on a 0.5% vertical agarose gel stained with ethidium bromide.* The approximate molecular masses (in kilobases, kb) of the plasmids are indicated by the numbers on the right side with arrows indicating the bands to which each corresponds; "LDE" indicates a plasmidlike linear DNA element. The bracket (chr) indicates the region in which the chromosomal DNA is found.

mal DNA bands. Approximate sizes for the plasmid molecules were reported elsewhere (Faust et al., 1983; Gonzalez and Carlton, 1984;

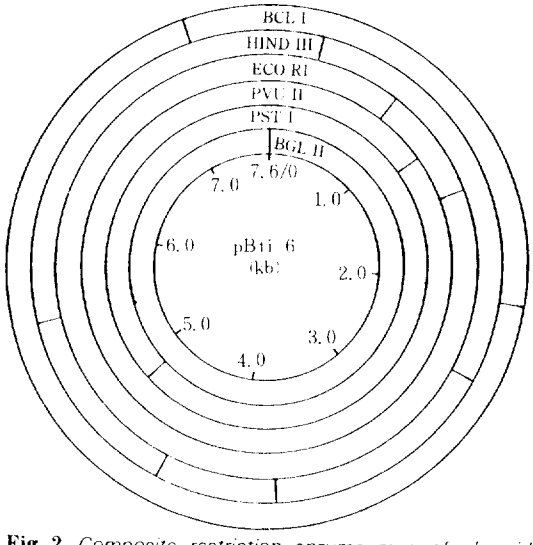

Fig. 2. *Composite restriction enzyme map of plasmid pBti6 isolated from Bacillus thuringiensis serovar israelensis 4Q1.* The map is oriented with respect to the single Bgl II site and is caliberated in kilobases (kb).

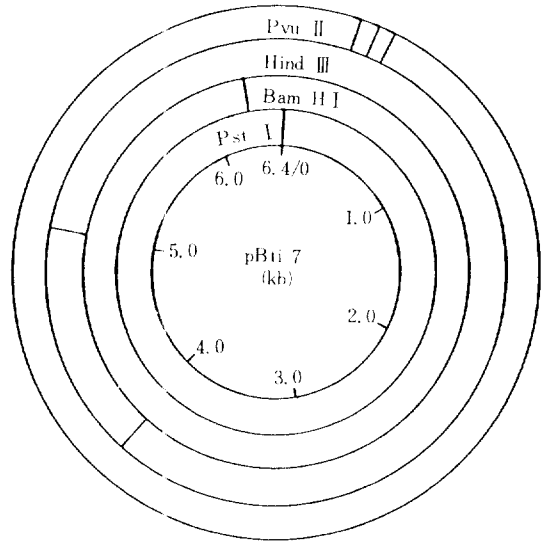

Fig. 3. *Composite restriction enzyme map of plasmid pBti7 isolated from Bacillus thuringiensis serovar israelensis 4Q1.* The two small restriction fragments generated by *Pvu* II are ambiguous with respect to each other. The single *Pst* I site was selected as the reference point. The map is calibrated in kilobases (kb).

Ward et al., 1984). Eight of the bands in the plasmid pattern are covalently closed-circular (ccc) plasmid molecules with estimated sizes of ~204, ~167, 109, 16, 7.6, 6.4, and 5.0 kilobases (kb). The remaining band (LDE) is a plasmid-like linear DNA element. The latter 3 ccc plasmids (designated pBti6, pBti7, and pBti8 respectively) were isolated from the gels and analyzed with nine restriction endonuclease enzymes as described in the Materials and Methods section.

Composite restriction enzyme maps

Digests of pBti6 revealed a single site for *Bgl*II, *Pst* I, and *Pvu* II, generating linear 7.6 kb molecules, two sites for *Eco* RI and *Bcl* I, and five sites for *Hind* III (Fig. 2). All other enzymes tested failed to cleave pBti6. The plasmid pBti7 contained one restriction site for *Bam* HI and *Pst* I and after digestion resulted in linear plasmid forms of 6.4 kb molecules. On *Hind* III digestion, two restriction fragments were obtained of M_r 5.3 and 1.0 kb, respectively. After *Pvu* II digestion, three restriction fragments of M_r 6.2, 0.07, and 0.07 kb were obtained, respectively. The two smaller fragments co-migrated and are ambiguous with respect to each other in the map (Fig. 3). Restric-

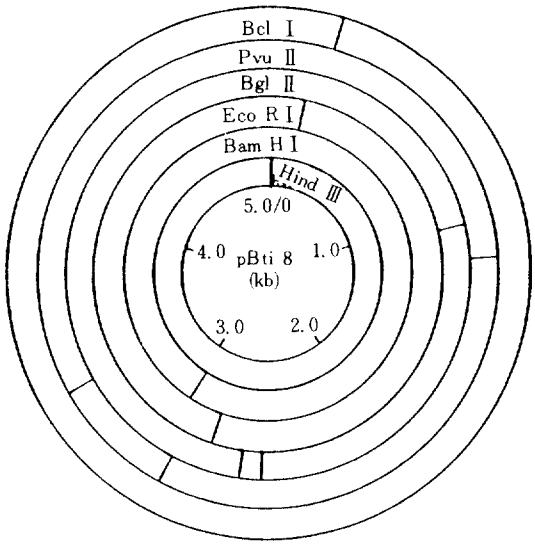

Fig. 4. *Composite restrication enzyme map of plasmid pBti8 isolated from Bacilus thuringiensis serovar israelensis 4Q1.* The map is oriented with respect to the single *Hind* III site and is calibrated in kilobases (kb).

tion enzymes *Bcl* I, *Bgl* II, *Eco* RI, *Kpn* I, and *Sal* I did not digest pBti7. Plasmid pBti8 (Fig. 4) cut with *Bam* HI, *Bcl* I, and *Hind* III produced one fragment each (5 kb) and with *Eco* RI generated two bands (2.7, 2.3 kb). Digestion of pBti8 with *Bgl* II and *Pvu* II resulted in 3 bands each ranging in size from 0.08 to 3.4 kb. *Kpn* I, *Pst* I, and *Sal* I failed to cleave pBti8. The construction of the maps were mainly facilitated by the various enzymes which cleave the plasmids at one, two, and in some cases three sites. The sites were unambiguously positioned relative to each other by all combinations of double digests between the appropriate restriction enzymes. To ensure that the first restriction enzyme digest had gone to completion before proceeding with the second enzyme, samples of the primary digest were included in the gel at adjacent slots in addition to the markers; small differences in migration were then readily detected. In order to compare the sizes of partial and complete-digestion products and to deduce which fragments might to adjacent to one another in the original plasmid, partical digests were carried out where deemed useful. The maps were built up from the data by a trial and error process in addition to basic logic. Table 1 summarizes the sizes of *B. thuringiensis* serovar *israelensis* plasmid DNA fragments obtained by digestion with restriction endonucleases *Bam* HI,

Table 1. *Summary of Bacillus thuringiensis serovar israelensis plasmid DNA fragments obtained by digestion with restriction endonucleases Bam HI, Bcl I, Bgl II, Eco R I, Hind III, Kpn I, Pst I, Pvu II and Sal I.*

Enzyme	Fragment Size (kb)	Sum	Total Number of Fragments Generated
	pBti6		
Bam H I	–	–	0
Bcl I	5.1, 2.5	7.6	2
Bgl II	7.6	7.6	1
Eco R I	6.9, 0.7	7.6	2
Hind III	2.4, 2.3, 1.2, 1.0, 0.7	7.6	5
Kpn I	–	–	0
Pst I	7.6	7.6	1
Pvu II	7.6	7.6	1
Sal I	–	–	0
	pBti7		
Bam H I	6.4	6.4	1
Bcl I	–	–	0
Bgl II	–	–	0
Eco R I	–	–	0
Hind III	5.3, 1.0	6.3	2
Kpn I	–	–	0
Pst I	6.4	6.4	1
Pvu II	6.2, 0.07, 0.07	6.3	3
Sal I	–	–	0
	pBti8		
Bam H I	5.0	5.0	1
Bcl I	5.0	5.0	1
Bgl II	3.4, 1.5, 0.08	5.0	3
Eco R I	2.7, 2.3	5.0	2
Hind III	5.0	5.0	1
Kpn I	–	–	0
Pst I	–	–	0
Pvu II	2.8, 1.7, 0.4	4.9	3
Sal I	–	–	0

Heading

Cleavage maps of *B. thuringiensis* plasmids.

Bcl I, *Bgl* II, *Eco* RI, *Hind* III, *Kpn* I, *Pst* I, *Pvu* II and *Sal* I.

For a plasmid molecule to be useful as a cloning vector it is desirable that it contains a sigle restriction site. At present the cloning possibilities for these replicons reside primarily in the single restriction enzyme sites determind for pBti6 (*Bgl* II, *Pst* I and *Pvu* II), pBti7 (*Bam* HI and *Pst* I), or pBti8 (*Bam* HI, *Bcl* I and *Hind* III). Generally, the recognition sites mapped differ either in length or nucleotide sequence and these sites are randomly distributed within the plasmid DNA's. Further studies are required to determine the usefulness of these single-hit restriction sites and to facilitate their usefulness as cloning vehicles. Further studies on plasmid DNA's indigenous to *B. thuringiensis* serovar *israelensis* are in progress.

적 요

Bacillus thuringiensis serovar *israelensis* 4Q1 로 부터 분자량이 204, 167, 109, 103, 16, 7.6, 6.4, 5.0 Kb인 8 개의 Plasmid 가 분리되었다. 이 Plasmid들 가운데 3 개의 작은 Plasmid들을 pBti6, pBti7, pBti8로 명시하였으며 이 Plasmid들은 크기가 작고 분리하기가 용이한 점에서 Cloning vector로써 적합하리라고 생각된다. 이 Plasmid들의 제한효소지도를 작성키위하여 9 종의 효소를 사용한바 그 가운데 7 종의 효소는 Plasmid DNA를 1-5 개 부위에서 절단하였다. 제한효소에 의한 절단부위를 Plasmid별로 살펴보면 pBti6의 경우는 *Bgl* II, *Pst* I 그리고 *Pvu* II에 의해서는 1 개부위, *Bcl* I 과 *Eco* RI에 의해서는 2 개부위, 그리고 *Hind* III로서는 5 개부위에서 절단되었고 Plasmid pBti7 은 *Bam* HI 과 *Pst* I 로서는 1 개부위, *Hind* III로는 2 개부위, 그리고 *Pvu* II에 의해서는 3 개부위에서 절단되었다. Plasmid pBti8을 보면 *Bam* HI, *Bcl* I 그리고 *Hind* III에 의해서는 1 개부위, *Eco* RI 로는 2 개부위, 그리고 *Bgl* II와 *Pvu* II에 의해서는 3 개부위가 절단되었다. 이와같이 제한효소에 의한 Plasmid DNA절단에 의해서 Plasmid pBti6, pBti7 pBti8 의 혼합 제한효소지도가 작성되었고 Plasmid DNA의 절단부위는 효소에 의한 1-2 혹은 부분절편들에 의해서 결정되었다. 한편 모든 절단부위의 위치는 *Bgl* II (pBti6), *Pst* I (pBti7) 그리고 *Hind* III (pBti8)에 의한 단일절단 부위들에 관련해서 각기 배열되었다.

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