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Detection of *cryIB* Genes in *Bacillus thuringiensis* subsp. *entomocidus* and subsp. *subtoxicus*

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To find new crystal protein genes, we screened 42 *Bacillus thuringiensis* strains of serovar standards by Southern hybridization with a *cryI*-specific probe which was amplified from *B. thuringiensis* subsp. *kurstaki* HD1 by polymerase chain reaction (PCR). Two strains, *B. thuringiensis* subsp. *entomocidus* HD9 and subsp. *subtoxicus* HD109, generated weak signals under the low-stringency hybridization conditions. Further analysis with Southern hybridization revealed that the two strains contained *cryIB* genes which are slightly different from those of *B. thuringiensis* subsp. *thuringiensis* HD2. These results were confirmed by PCR with *cryIB*-specific primers followed by the restriction analysis of PCR products.

Bacillus thuringiensis is a gram-positive bacterium that produces crystalline inclusions composed of proteins which have a highly specific insecticidal activity against the larvae of certain members of lepidopteran, dipteran or coleopteran species. The insecticidal crystal proteins, encoded by *cry* genes, have been classified as CryI, II, III, IV, and V, depending on the host specificity and the degree of amino acid homology (7). Since the lepidopteran species susceptible to CryI proteins are agronomically important pests, major research attentions have centered on the *cryI* gene class consisting of several subclasses: *cryIA, cryIB, cryIC, cryID, cryIE, cryIF* and *cryIG*.

Increasing restrictions on the use of existing chemical insecticides in many countries, the pest resistance problems, and the rapidly rising costs of developing new synthetics in an increasingly stringent regulatory environment stimulated the agrochemical industry to develop viable and environmentally acceptable alternatives. Because *B. thuringiensis* has been used commercially for more than two decades without reports of substantial resistance in open-field populations of insect pests, it is becoming increasingly important for the management of pests. However, recent reports presented a resistance to *B. thuringiensis* subsp. *kurstaki* in the diamondback moth (4, 15) and indian meal moth (9). For managing the development of resistance to *B. thuringiensis* toxins, several methods had been tried (13). One successful discovery was that resistant strain of indian meal moth to a CryIA(b) toxin which is correlated with a 50-fold reduction in affinity of the membrane receptor is sensitive to a second type of toxin, CryIC, that apparently recognizes a different receptor (14). This report stimulated a search for novel CryI toxin although its insecticidal activity is less strong than previously known CryI toxins.

Methods for the screening of novel toxin have been established on the basis of Southern blot analysis (11), reactivity with various monoclonal antibodies (8), or polymerase chain reaction (PCR) followed by restriction analysis of the PCR products (6, 10). In this report, we screened 42 *B. thuringiensis* strains of serovar standards by using Southern hybridization with *cryI*-specific probe which was amplified from *B. thuringiensis* subsp. *kurstaki* HD1 by PCR. The results showed that two strains, *B. thuringiensis* subsp. *entomocidus* HD9 and subsp. *subtoxicus* HD109, include *cryIB* genes different at least one restriction enzyme site from that of *B. thuringiensis* subsp. *thuringiensis* HD2. The distribution of *cryIB* gene was reported only one case in *B. thuringiensis* subsp. *thuringiensis* HD2. We present here an additional two strains carrying the *cryIB* genes which are slightly different from the known *cryIB* gene.

MATERIALS AND METHODS

Bacterial Strains

B. thuringiensis strains were obtained from the *Bacillus*

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Genetic Stock Center, Ohio, U.S.A. and Korean Collection for Type Cultures, Genetic Resources Center, Genetic Engineering Research Institute, KIST, and they are listed in Table 1.

Table 1. Description of *Bacillus thuringiensis* serovars used in this study.

	Serovar or biovar	HD or strain no.	Serovar designation
1	Aizawai	HD11	7
2		HD 137	7
3	Alesti	HD 4	3a
4		NRRL 4041	3a
5	Canadensis	HD 224	4a4c
6		NRRL 4056	
7	Colmeri	IS 720	20
8		HD 847	20
9	Darmstadiensis	IPL	10
10		HD 146	10
11	Dakota	HD 511	16
12	Dendrolimus	HD 7	4a4b
13	Entomocidus	HD 635	6
14		HD 9	6
15	Entomocidus-limassol	NRRL 4047	6
16	Finitimus	HD 3	2
17	Galleriae	HD 29	5a5b
18		NRRL 4045	5a5b
19	Indiana	HD 521	15
20	Israelensis	IPS 82	14
21		HD 567	14
22	Kenya	HDB 23	4a4c
23	Kumamotoensis	HD 867	18
24	Kurstaki	HD 1	3a3b
25		Dipel	3a3b
26		HD 73	3a3b
27	Kyushuensis	HD 541	11a11c
28	Morrisoni	HD 12	8a8b
29		HD 116	8a8b
30		NRRL 4049	8a8b
31	Ostriniae	HD 501	8a8c
32	Parkistani	HD 395	13
33	Sandiego		8a8b
34	Sotto		4a4b
35	Subtoxicus	HD 109	6
36	Thompsoni	HD 542	12
37	Thuringiensis	HD 2	1
38	Tochigiensis	HD 868	19
39	Tohokuensis	4V1	17
40	Tolworthi	HD 537	9
41		NRRL 4050	9
42	Toumanoffi	HD 201	11a11b

Dot Blot Analysis

Total *B. thuringiensis* DNA was prepared by the method of Kalman *et. al.* (10). After the DNA samples were dotted on the nylon membrane, the membrane was wetted in a denaturation buffer (0.5 N NaOH, 1.5 M NaCl) for 5 min, then in a neutralization buffer (1 M Tris·Cl, pH 7.5, 1.5 M NaCl) for 15 min. Then the DNAs on the wet membrane were fixed by UV-crosslinking, and were used for hybridization. The hybridization probe which was amplified from *B. thuringiensis* subsp. *kurstaki* HD1 by PCR with *cryI*-specific primers published elsewhere (3), Lep2A; 5'-CCGAGAAAGTCAAACATGCG-3' and Lep2B; 5'-TACATGCCCTTTCACGTTCC-3', was labelled by DIG-DNA Labeling Kit from Boehringer Mannheim. Hybridization was performed under high- or low-stringency conditions and washing. In the high-stringency condition, hybridization was performed at 68°C in a standard prehybridization buffer [5X SSC(750 mM NaCl, 75 mM sodium citrate, pH 7.0), 1% blocking reagent from Boehringer Mannheim, 0.1 % N-lauroylsarcosine and 0.02% sodium dodecyl sulfate (SDS)] and washing was performed in 2X SSC , 0.1% SDS at room temperature followed by washing in 0.1X SSC, 0.1% SDS at 68°C. In the low-stringency condition, hybridization was performed at 37°C in a modified prehybridization buffer (3X SSC, 1% blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS and 50% formamide) and washing was performed in 2X SSC, 0.1% SDS at room temperature followed by washing in the same buffer at 50°C. Detection was performed by a DIG-DNA Detection Kit from Boehringer Mannheim.

Southern Blot Analysis

After the digestion of total DNAs with various restriction enzymes, the samples were electrophoresed and transferred onto the nylon membrane by Trans-Vac, TE 80 from Hoefer Scientific Instrument. Hybridization was performed under low-stringency conditions with the same probe as described above.

Polymerase Chain Reaction

CryIB-specific primers were designed with the following sequences: IB-a, 5'-TTTAGCGCTTCATCACGATGGAGTAA-3'; IB-b, 5'-TTTCAATCTTCTTGGATTCGTATTAG-3'. Amplification was accomplished with the DNA Thermal Cycler (Perkin Elmer Cetus) by using the Step-Cycle program set to denature at 94°C for 45 sec, anneal at 45°C for 45 sec, and extend at 72°C for 1 min, followed by a 4-s-per-cycle extension for a total of 35 cycles.

RESULTS AND DISCUSSION

Dot Blot Analysis

Because the search of novel *cry* genes by the method

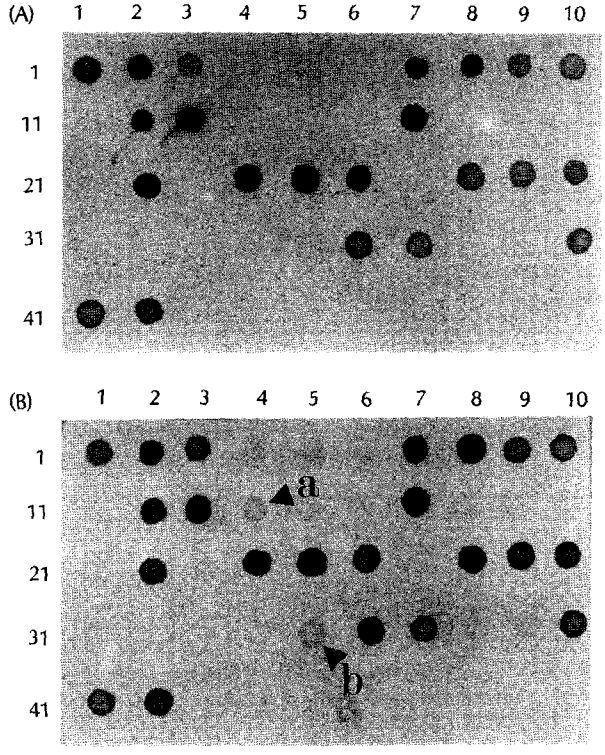

Fig. 1. Dot blot analysis of the serovar standard strains of *B. thuringiensis* at the high-stringency(A) and low-stringency condition(B).

Strain numbers put in order of description in Table 1. Arrows indicate faint hybridization signals at the low-stringency condition: a, *B. thuringiensis* subsp. *entomocidus* HD9; b, subsp. *subtoxicus* HD109.

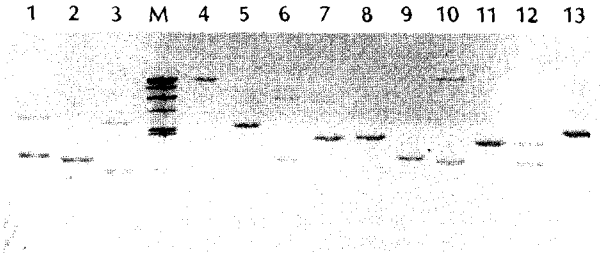

Fig. 2. Southern blot analysis of *B. thuringiensis* subsp. *entomocidus* HD9.

Total DNAs were digested with: lanes 1, *PvuII* and *DraI*; 2, *NciI*; 3, *EcoRI* and *HindIII*; 4, *PvuII*; 5, *PvuII* and *BglII*; 6, *PvuII* and *ClaI*; 7, *PvuII* and *EcoRV*; 8, *PvuII* and *KpnI*; 9, *PvuII* and *PstI*; 10, *PvuII* and *StuI*; 11, *PvuII* and *XbaI*; 12, *PvuII* and *XhoI*. The DNAs were hybridized and detected with the methods described in MATERIALS AND METHODS. M is lambda DNA digested with *HindIII*.

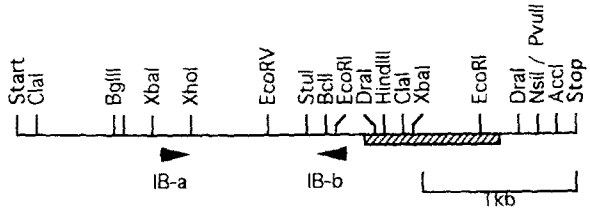

Fig. 3. Restriction map of *cryIB* gene in *B. thuringiensis* subsp. *thuringiensis* HD2.

Dashed bar indicates the hybridized region with probe sequences. *cryIB*-specific primers situated below the map by arrows.

of Southern blot analysis is based on homologous sequences, finding *cry* genes with low homologous to known genes is somewhat difficult. However, it was reported that a *cryV* gene was found with a probe of *cryIIIA* gene by low-stringency Southern hybridization analysis (16). On the basis of this report, we tried to find novel *cry* genes in the 42 serovar standard strains of *B. thuringiensis* by the method of Southern hybridization. Hybridization probe, amplified from the *B. thuringiensis* subsp. *kurstaki* HD1 by PCR, is highly homologous with *cryIA*(a), *IA*(b), *IA*(c), *ID*, *IE*, and *IF* genes, and slightly less homologous with *cryIB* and *IC* genes. As shown in Fig. 1, faint hybridization signals generated in *B. thuringiensis* subsp. *entomocidus* HD9 and subsp. *subtoxicus* HD109 under low-stringency hybridization (Fig. 1, B) conditions. These two strains were bioactive against *Plutella xylostella* but not against *Bombyx mori* (data not shown). According to the published data, Cry proteins which were bioactive against *P. xylostella* but not against *B. mori* were CryIA(b), IA(c) and IB proteins (5). Among these, genes encoding CryIA(b) and IA(c) proteins are highly homologous with the hybridization probe used in this study. Thus, the two strains may contain derivatives of *cryIB* gene or novel crystal protein genes which are less homologous with the known *cry* genes.

Southern Blot Analysis

For the investigation of detailed restriction maps of *cry* genes in *B. thuringiensis* subsp. *entomocidus* HD9 and subsp. *subtoxicus* HD109, Southern hybridization was performed with the same probe used in dot blot analysis under low-stringency conditions. Since the location of *PvuII* site was identified at the downstream of the probe binding region by preliminary experiment (data not shown), total DNAs of *B. thuringiensis* subsp. *entomocidus* HD9 were double digested with *PvuII* and various restriction enzymes described in Fig. 2. As a hybridization result, the deduced restriction map of the *cry* gene in *B. thuringiensis* subsp. *entomocidus* HD9 was identical with the *cryIB* gene in *B. thuringiensis* subsp. *thuringiensis* HD2 (Fig. 3). But the site of one restriction enzyme, *ClaI*, at the N-terminal region of coding sequence seemed to be absent in *B. thuringiensis* subsp. *entomocidus* HD9, and it is discriminated with *cryIB* gene in *B. thuringiensis* subsp. *thuringiensis* HD2. In *B. thuringiensis* subsp. *subtoxicus* HD109, total DNAs were double digested with various enzymes and *NsiI* or *StuI* whose sites are located at the downstream or upstream of the probe binding region, respectively. As shown

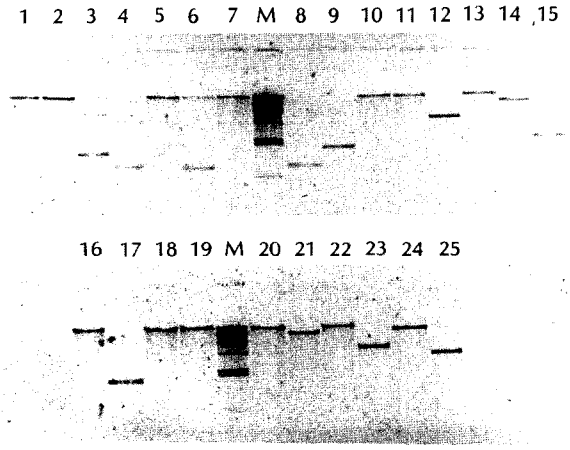

Fig. 4. Southern blot analysis of *B. thuringiensis* subsp. *subtoxicus* HD109.
Total DNAs were digested with: lanes 1, *Nsi*I; 2, *Nsi*I and *Bam*HI; 3, *Nsi*I and *Bcl*I; 4, *Nsi*I and *Cla*I; 5, *Nsi*I and *Nco*I; 6, *Nsi*I and *Pst*I; 7, *Nsi*I and *Pvu*I; 8, *Nsi*I and *Eco*RI; 9, *Nsi*I and *Eco*RV; 10, *Nsi*I and *Sac*I; 11, *Nsi*I and *Sal*I; 12, *Nsi*I and *Sca*I; 13, *Nsi*I and *Sma*I; 14, *Nsi*I and *Sph*I; 15, *Nsi*I and *Xho*I; 16, *Stu*I; 17, *Stu*I and *Acc*I; 18, *Stu*I and *Bam*HI; 19, *Stu*I and *Nco*I; 20, *Stu*I and *Pvu*I; 21, *Stu*I and *Sac*I; 22, *Stu*I and *Sal*I; 23, *Stu*I and *Sca*I; 24, *Stu*I and *Sma*I; 25, *Stu*I and *Sph*I. The DNAs were hybridized and detected with the methods described in MATERIALS AND METHODS. M is lambda DNA digested with *Hin*dIII.

in Fig. 4, the hybridization result was identical with *B. thuringiensis* subsp. *entomocidus* HD9. In both strains, additional signals corresponding to other *cry* genes were not found. These results suggested that each of the two strains included only one *cry* gene. Since only one case of the strain containing *cryIB* gene was reported in *B. thuringiensis* subsp. *thuringiensis* HD2 (2), we present here the additional two strains harboring *cryIB* genes.

Polymerase Chain Reaction

To confirm the presence of *cryIB* genes in the two strains, PCR was performed with *cryIB*-specific primers, and *B. thuringiensis* subsp. *thuringiensis* HD2 was used as a reference. The size of PCR products was about 1 kb, and it was the same in the three strains (Fig. 5). Also, following restriction analysis with *Eco*RV or *Xho*I was the same result in the three strains. These results confirmed that *B. thuringiensis* subsp. *entomocidus* HD9 and subsp. *subtoxicus* HD109 contain the *cryIB* genes.

For the search of novel crystal protein genes, many researchers depend on the bioassay. However, the bioassay against various insects is laborous and time-consuming when a large number of strains are tested. Difficulty in the search for novel crystal protein genes by bioassay is also faced when the activity of novel crystal protein is masked by that of previously known crystal proteins. Moreover, if the novel crystal protein

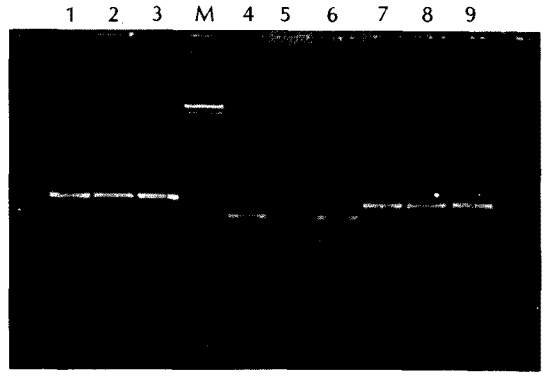

Fig. 5. Polymerase chain reaction and following restriction analysis.
Total DNA samples were analyzed by PCR with cryIB-specific primers from: lanes 1, *B. thuringiensis* subsp. *thuringiensis* HD2; 2, subsp. *entomocidus* HD9; 3, subsp. *subtoxicus* HD109. Following restriction analysis of the PCR products were performed. Lanes 4~6 are *Eco*RV digested PCR products from HD2 (lane 4), HD9 (lane 5) and HD109 (lane 6). Lanes 7~9 are *Xho*I digested PCR products from HD2 (lane 7), HD9 (lane 8) and HD109 (lane 9). M is lambda DNA digested with *Hin*dIII.

gene is not expressed in original strain, that is a silent gene, it cannot be found by the bioassay. Overcoming the problems mentioned above, we recommend that the search for novel crystal protein genes be tried on the DNA basis by the method of Southern hybridization used in this study. The advantage of these methods is not only that many samples are dealt with but also that silent genes can be found.

It was reported that *B. thuringiensis* subsp. *thuringiensis* has a stronger insecticidal activity against some lepidopteran species such as *Pieris brassicae* than *B. thuringiensis* subsp. *kurstaki* HD1 which is the most commercial preparations (1). But the practical application of *B. thuringiensis* subsp. *thuringiensis* has been restricted because it produces a β-exotoxin (12). The β-exotoxin is not a very host-specific because it inhibits the RNA synthesis interfering with the DNA-dependent RNA polymerases, play a key role in transcription, present in the cells of every organism. Therefore, preparations containing β-exotoxin may not be used in open-fields because of the possible destruction of ecosystem. It was reported that the two strains presented in this study do not produce a β-exotoxin (12). Thus, instead of *B. thuringiensis* subsp. *thuringiensis*, the two strains may be used as a biocontrol agent against some lepidopteran species without threatening the environment.

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