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# Identification of Novel *cry*-Type Genes from *Bacillus thuringiensis* Strains on the Basis of Restriction Fragment Length Polymorphism of the PCR-Amplified DNA

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**Two pairs of universal oligonucleotide primers were designed to probe the most conserved regions of all known *cryI*-type gene sequences so that the amplified PCR fragments of the DNA template from *Bacillus thuringiensis* strains may contain all possible *cryI*-type gene sequences. The restriction fragment length polymorphism (RFLP) patterns of the PCR-amplified fragments revealed that 14 distinct *cry*-type genes have been identified from 20 *B. thuringiensis* strains. Those *cry*-type genes included *cryIA(a)*, *cryIA(a)*†, *cryIA(b)*, *cryIA(b)*†, *cryIA(c)*, *cryIB*†, *cryIC*, *cryIC*†, *cryIC(b)*, *cryID*, *cryIE*, *cryIF*, *cryIF*†, and *cryIII*† (a dagger at the end of a gene designation indicates a novel *cry*-type gene determined by restriction mapping or DNA sequences). Among them, the sequences of *cryIA(a)*†, *cryIA(b)*†, *cryIB*†, *cryIC*†, *cryIF*†, and *cryIII*† were found to be different from the corresponding published *cry* gene sequences. Interestingly, five *cry*-type genes [*cryIA(a)*-, *cryIB*-, *cryIC*-, *cryIC(b)*-, and *cryIF*-type genes] and seven *cry*-type genes [*cryIA(a)*†-, *cryIA(b)*†-, *cryIB*†-, *cryIC*†-, *cryIC(b)*-, *cryIF*-, and *cryIII*-type genes] have been detected from *B. thuringiensis* subsp. *morrisoni* HD-12 and *B. thuringiensis* subsp. *wuhanensis*, respectively. Therefore, the PCR-RFLP typing system is a facile method to detect both known and novel *cry* genes existing in *B. thuringiensis* strains.**

*Bacillus thuringiensis* is a spore-forming gram-positive bacterium. During sporulation, the intracellular insecticidal crystal proteins (Cry proteins) are produced as phase-bright inclusions (7). These proteins are toxic to insect larvae in the orders *Lepidoptera*, *Diptera*, and *Coleoptera* (8). The Cry protein from *B. thuringiensis* has been developed as one of the most successful biological agents in industry to control insect pests (2).

The insecticidal crystal protein genes (*cry*) are normally associated with plasmids of large molecular mass (18). The insecticidal Cry proteins, encoded by *cry* genes, have been classified as CryI, -II, -III, or -IV, depending on the host specificity and the degree of amino acid homology (21).

*B. thuringiensis* soil isolates are distributed globally (12, 21, 25, 26). To obtain novel *B. thuringiensis* strains for the production of Cry proteins, the isolation of numerous new *B. thuringiensis* strains is becoming a routine activity in many industries. *B. thuringiensis* strains are classified into 34 serovars (16). As serotypes of the *B. thuringiensis* strains do not directly reflect the specific *cry* gene classes contained in the corresponding *B. thuringiensis* strains, prediction of insecticidal activity of a *B. thuringiensis* strain based on the serotyping seems impractical. Multiplex PCR is becoming an increasingly important method to identify the existence of *cry*-type genes (4, 10, 11, 23). However, the major drawback of this method is that it cannot identify the existence of a novel *cry* gene from a *B. thuringiensis* strain whose nucleotide sequence is unknown. The *cry*-type gene profiles (combinations) of many *B. thuringiensis* strains have not been identified (for a review, see reference 36); therefore, a new method should be developed to detect the novel *cry* genes in these *B. thuringiensis* strains.

In contrast to the multiplex PCR *cry* gene typing method, two pairs of universal oligonucleotide primers were designed to probe the most conserved regions of all known *cryI*-type gene sequences so that the amplified PCR fragments of the DNA template from *B. thuringiensis* strains may contain all possible *cryI*-type gene sequences. Following PCR amplification, restriction fragment length polymorphism (RFLP) was employed to identify the origin of the *cry*-type genes. By use of this PCR-RFLP *cry* gene typing system, 14 distinct *cry*-type genes from the tested *B. thuringiensis* strains were identified. Among them, six *cry*-type genes were found to have sequences different from the corresponding published *cry* gene sequences. Therefore, the PCR-RFLP typing system is a facile method to detect both known and novel *cry* genes existing in a *B. thuringiensis* strain. Applications of this PCR-RFLP method to the study of organization and differential expression of *cry* genes will be discussed.

## MATERIALS AND METHODS

**Bacterial strains and media.** *B. thuringiensis* strains, other than the strain isolated from Taiwan, i.e., YMB-82 (12), were collected from USDA Cotton Insect Research, Brownsville, Tex. Nutrient broth and agar, L broth, and L agar (LA) were from Difco.

**Isolation of DNA for PCR analysis.** A freshly (overnight) isolated colony incubated at 30°C on either a nutrient agar or LA plate was selected and restreaked on an LA plate. The plate was incubated at 37°C for 4 to 6 h. Cells (two loopfuls) from the LA plate were resuspended in 100 μl of lysis solution (10% sucrose, 50 mM Tris-HCl [pH 8.0], 20 mM EDTA, 1 mg of lysozyme per ml) in a 1.5-ml microcentrifuge tube. The remaining DNA extraction was done as described by Birnboim (3). Finally, the washed DNA pellets were resuspended in 20 μl of 1× Tris-EDTA (10 mM Tris-HCl [pH 8], 1 mM EDTA).

**Identification of *cry*-type genes by RFLP.** From a PC Gene multi-alignment analysis, three highly conserved regions from all published *cryI*-type genes were located. Four universal oligonucleotide primers, K5un2, K3un2, K5un3, and K3un3, were designed. Among them, the sequences of oligonucleotide primers K5un2 and K3un3 are complementary. The sequences of four oligonucleotide primers and the alignments of all published *cryI*-type gene sequences are shown in Table 1.

The sequence of oligonucleotide primer K5un2 is highly conserved with 10 *cryI*-type, 4 *cryIII*-type, 3 *cryIV*-type, and 1 *cryV*-type genes. The mismatch of the oligonucleotide primers among those sequences is less than 4 nucleotides (Table

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TABLE 1. Nucleotide sequences of oligonucleotide primers and nucleotide sequences of the corresponding regions of the distinct *cry* genes

| Primer or gene | Sequence[a] | Positions[b] | Reference |
|---|---|---|---|
| K5un2, K3un3[c] | AGGA-CCAGGATTTACAGGAGG | | |
| *cryIA(a)* | AGGA-CCAGGATTTACAGGAGG | 1994–2014 | 28 |
| *cryIA(b)* | AGGA-CCAGGATTTACAGGAGG | 1611–1631 | 34 |
| *cryIA(c)* | AGGA-CCAGGATTTACtGGtGG | 1854–1874 | 1 |
| *cryIB* | AGGA-CCAGGATTTACtGGtGG | 1554–1574 | 5 |
| *cryIC* | AGGA-CCAGGATTTACAGGAGG | 1504–1524 | 22 |
| *cryIC(b)* | AGGt-CCAGGATTTACAGGAGG | 1753–1773 | 23 |
| *cryID* | AGGt-CCtGGATTTACAGGtGG | 1700–1720 | 15 |
| *cryIE* | AGGA-CCAGGATTTACAGGAGG | 1578–1598 | 33 |
| *cryIF* | AGGg-CCcGGgTTTACgGGAGG | 1926–1946 | 13 |
| *cryIG* | AtGAtCCAGGATTTAtAGGAGG | 2138–2159 | 30 |
| *cryIIIA* | AGGt-CCtaGgTTTACAGGAGG | 1608–1628 | 20 |
| *cryIIIB* | AGGt-CCAGGATTcACAGGAGG | 1617–1637 | 29 |
| *cryIIIBB* | AGGt-CCAGGATTcACAGGAGG | 1606–1626 | 17 |
| *cryIIIC* | AGGg-CCtGGATTTACAGGtGG | 1730–1750 | 24 |
| *cryIVA* | AGGA-CCtGGtcaTACAGGAGG | 1671–1691 | 35 |
| *cryIVB* | gGGA-CCtGGtcaTACAGGgGG | 1653–1673 | 14 |
| *cryIVC* | AGGt-CCtGGtcacACAGGtGG | 2527–2547 | 32 |
| *cryV* | AGGA-CCAGGATTTACAGGtGG | 1932–1952 | 31 |
| K3un2 | GCTGTGACACGAAGGATATAGCCAC | | |
| *cryIA(a)* | GCTGTGACACGAAGGATATAGCCAC | 3603–3627 | 28 |
| *cryIA(b)* | GCTGTGACACGAAGGATATAGCCAC | 3143–3167 | 34 |
| *cryIA(c)* | GCTGTGACACGAAGGATATAGCCAC | 3470–3494 | 1 |
| *cryIB* | GCTGTGACACGAAGGATATAGCCAC | 3215–3239 | 5 |
| *cryIC* | GCTGTGACACGAAGGATATAGCCAC | 3150–3174 | 22 |
| *cryIC(b)* | GCTGTaACACGAAGGATATAGCCAC | 3360–3384 | 23 |
| *cryID* | GCTGTaACACGAAGGATATAGCCAC | 3295–3319 | 15 |
| *cryIE* | GCTGTaACACGAAGGATATAGCCAC | 3188–3212 | 33 |
| *cryIF* | GCTGTGACACGAAGGATATAGCCAC | 3536–3560 | 13 |
| *cryIG* | GCTGTtACACGtAatAcATAtttAC | 3730–3754 | 30 |
| *cryIIIC* | GCTGTtACAtGtAaGATATAcCCAC | 3335–3359 | 24 |
| K5un3 | CAATGCGTACCTTACAATTGTTTAAGTAAT | | |
| *cryIA(a)* | gAATGCaTtCCTTAtAATTGTTTAAGTAAc | 551–580 | 28 |
| *cryIA(b)* | gAATGCaTtCCTTAtAATTGTTTAAGTAAc | 166–195 | 34 |
| *cryIA(c)* | gAATGCaTtCCTTAtAATTGTTTAAGTAAc | 412–441 | 1 |
| *cryIC* | CAATGCaTACCTTACAATTGTTTAAGTAAT | 68–97 | 22 |
| *cryIC(b)* | CAATGCGTACCTTACAATTGTTTAAGTAAT | 317–346 | 23 |
| *cryID* | CAATGtGTgCCTTACAATTGTTTAAGTAAT | 286–315 | 15 |
| *cryIE* | CAATGCGTgCCTTAtAATTGTTTAAaTAAT | 154–183 | 33 |
| *cryIF* | CAATGCGTACCTTACAATTGTTTAAaTAAT | 499–528 | 13 |

[a] Sequences that do not match those of oligonucleotide primers are shown by lowercase letters; gaps are shown by dashes.
[b] Position where the oligonucleotide primers probe the *cry*-type gene.
[c] Oligonucleotide primer K3un3 is complementary to K5un2.

1). K3un2 is highly conserved with all *cryI*-types genes and has only a 5-nucleotide mismatch with the *cryIIIC* gene. The oligonucleotide primer K5un3 is specially designed for the amplification of *cryIC*, *cryIC(b)*, *cryID*, *cryIE*, and *cryIF* genes; therefore, this sequence is distantly related to all other *cry* gene sequences (Table 1).

Oligonucleotide primers K5un2 and K3un2 were used to perform 30 PCR thermal cycles (94°C for 1 min, 52°C for 2 min, and 72°C for 1.5 min) with template DNA from different *B. thuringiensis* strains. In theory, all template DNAs containing *cryIA* to *cryIG* and *cryIIIC* genes should produce a PCR fragment with a size of about 1.6 kb (PCR-1). After *Pst*I and *Xba*I double digestion of the PCR products, the *cry*-type genes of the corresponding *B. thuringiensis* strains were identified by their RFLP patterns. The predicted sizes of PCR fragments and their RFLP patterns are listed in Table 2. The strategies for PCR amplifications and RFLP analysis of the DNA templates from *B. thuringiensis* strains are shown in Fig. 1. For PCR amplifications, 1 to 5 μg of total DNA per ml or 0.1 to 0.5 μg of plasmid DNA per ml was used as the template DNA. The concentration of each oligonucleotide primer used for PCR amplification was 0.2 μM. Five units of *Taq* from TaKaRa Shuzo Co., Ltd., was used in a 100-μl PCR mixture. Amplification was performed with the DNA thermal cycler 480 from Perkin-Elmer Corp.

Some *cry*-type genes such as *cryIC*, *cryIC(b)*, *cryID*, *cryIE*, and *cryIF* were not easily identified as a result of the similarity of their RFLP patterns. To overcome this problem, the specific oligonucleotide primers K5un3 and K3un3 were used. The size of the PCR product amplified by this pair of oligonucleotide primers is about 1.4 kb (PCR-2). Their predicted RFLP patterns and the strategies for *cry*-type gene analysis are shown in Table 2 and Fig. 1, respectively.

**Cloning of PCR products.** All PCR products were treated with Klenow fragment to fill in any recessed ends before ligation to the *Sma*I or *Hin*cII sites of pUC18.

**Other methods.** The standard molecular and biochemical methods used have been described previously (27). Restriction enzymes, Klenow fragment, and T4 DNA kinase were purchased from Boehringer Mannheim. All enzymes were used as discussed in the instructions of the manufacturers.

## RESULTS

**Identification of *cryI*- and *cryIII*-type genes from *B. thuringiensis* strains.** DNA templates from 20 distinct *B. thuringiensis* strains were used for PCR amplification with universal oligonucleotide primers K5un2 and K3un2. All tested DNA templates produced 1.6 to 1.7-kb PCR products (Fig. 2A). In addition, templates from *B. thuringiensis* subspecies *thompsoni*, *finitimus*, *canadensis*, *pakistani*, *kyuchuensis*, *tohokuensis*, *israelensis*, and *tolworthi* also produced some PCR products larger or smaller than 1.6 kb (Fig. 2A).

The results of RFLP pattern analysis after *Pst*I and *Xba*I digestion of the PCR products are shown in Fig. 2B to D. The sizes of the restriction fragments and the predicted *cry*-type

TABLE 2. Predicted sizes of PCR products and the RFLP of various *cry*-type genes

| *cry*-type gene | PCR-1 (K5un2-K3un2) | | PCR-2 (K5un3-K3un3) | |
|---|---|---|---|---|
| | Predicted size (bp) of PCR product | Predicted sizes (bp) of PCR products digested with *Pst*I and *Xba*I | Predicted size (bp) of PCR product | Predicted size(s) (bp) of PCR product(s) digested with *Eco*RI and *Pst*I |
| *cryIA(a)* | 1,635 | 1,117, 518 | 1,463 | 726, 493, 244 |
| *cryIA(b)* | 1,557 | 1,039, 518 | 1,465 | 726, 496, 244 |
| *cryIA(c)* | 1,641 | 322, 801, 518 | 1,463 | 726, 434, 244, 59 |
| *cryIB* | 1,686 | 1,015, 16, 655 | NP[a] | NP |
| *cryIC* | 1,671 | 239, 758, 16, 140, 95, 423 | 1,457 | 1,457 |
| *cryIC(b)* | 1,632 | 958, 518, 140, 16 | 1,457 | 1,196, 261 |
| *cryID* | 1,620 | 962, 140, 518 | 1,436 | 819, 310, 307 |
| *cryIE* | 1,635 | 218, 743, 16, 140, 518 | 1,445 | 986, 238, 221 |
| *cryIF* | 1,635 | 961, 16, 140, 518 | 1,448 | 879, 548, 21 |
| *cryIG* | 1,618 | 377, 1,241 | NP | NP |
| *cryIIIC* | 1,629 | 589, 525, 515 | NP | NP |

[a] NP, no product.

genes of the 20 distinct *B. thuringiensis* strains are listed in Table 3. The RFLP patterns of the PCR products from *B. thuringiensis* subspecies *thuringiensis* HD-2, *kurstaki* HD-73, and *aizawai* HD-133 and *B. thuringiensis* isolate YMB-82 matched the predicted restriction patterns listed in Table 2. Most importantly, the *cry*-type gene combinations (Table 3) detected by the RFLP patterns of those *B. thuringiensis* strains were identical to that of the published results of the multiplex PCR method (11). Thus, these results indicated that the PCR-RFLP is a reliable method to identify *cry*-type genes. The *cry*-type gene combination of *B. thuringiensis* subsp. *kenyae* deduced from the RFLP pattern was similar to that of the published data (36). In addition, we detected one more *cryIC(b)*-type gene in this strain (Table 3). Kalman et al. (23) showed that *B. thuringiensis* subsp. *galleriae* contains *cryIA(b)*, *cryIA(c)*, *cryIC(b)*, and *cryID* genes. However, in this *cry* gene typing method (Table 3), we detected only the *cryIA(a)*, *cryIC*, and *cryIC(b)* genes in this strain. The reason for these differences is not known. Perhaps the *B. thuringiensis* strains used in the two laboratories are different.

The *cry*-type gene combinations of *B. thuringiensis* subspecies *morrisoni* HD-12 and *wuhanensis* have not been reported previously. The RFLP patterns of the PCR products from these two strains were very complicated (Fig. 2B and D), indicating that these two strains should contain a considerable number of *cry*-type genes. The RFLP pattern of the PCR product from *B. thuringiensis* subsp. *morrisoni* HD-12 showed that this strain should contain *cryIA(a)*, *cryIC*†, *cryIC(b)*, and *cryIF* genes (Fig. 2B and D, Fig. 3B, and Table 3). Since we detected only a typical 655-bp *cryIB* fragment (Fig. 2D) from the RFLP pattern, the other typical *cryIB* fragment, of 1,015 bp, was missing. Therefore, we predicted that *B. thuringiensis* subsp. *morrisoni* HD-12 may contain a novel *cryIB* gene. There were at least five extra restriction fragments (Fig. 2B and D and Table 3), of 900, 800, 620, 240, 160, and 150 bp, that could not be identified from the predicted RFLP pattern (Table 2). This result probably implied that more novel *cry*-type genes may exist in *B. thuringiensis* subsp. *morrisoni* HD-12. However, we cannot rule out the possibility that these fragments may be derived from the nonspecific amplification of PCR products.

The RFLP pattern of the PCR-1 product from *B. thuringiensis* subsp. *wuhanensis* (Fig. 2 and Table 3) produced a pattern similar but not identical to the predicted restriction pattern of the *cryIA(a)*, *cryIA(b)*, and *cryIB* genes. Therefore, we speculated that these three genes should be different from those of the published *cry* gene sequences. This speculation was further confirmed by the restriction mapping of the PCR products (Fig. 4A). Although the restriction pattern of the *cryIC*-type gene in this strain (Fig. 2 and Table 3) was identical to that of the predicted restriction pattern (Table 2), the partial sequence of this gene (unpublished data) was not identical to that of the published *cryIC* gene. This result indicated that a novel *cryIC*-type gene may exist in *B. thuringiensis* subsp. *wuhanensis*. Two restricted fragments, of 1,200 and 620 bp (Fig. 2 and Table 3), from the RFLP pattern of this strain were not found in the predicted restriction pattern of *cry*-type genes (Table 2). This result probably suggests that some novel *cry*-type genes may exist in this strain.

The *cry*-type gene in *B. thuringiensis* subspecies *kumamotoensis*, *indiana*, *dakota*, *tohokuensis*, and *tochigiensis* was not determined (9). In contrast, from the analysis of the restriction patterns of the 1.6-kb PCR fragment (Fig. 2B), a typical RFLP pattern of a *cryIII*-type gene (Table 2) was detected from the PCR products of *B. thuringiensis* subspecies *kumamotoensis* and *dakota* (Fig. 2 and Table 3). However, the largest fragment, of 589 bp, of the predicted *cryIII*-type pattern (Table 2) was slightly smaller than the corresponding PCR-RFLP fragment, which was 620 bp (Fig. 2 and Table 3), of these two strains. The partial nucleotide sequence of the PCR product (unpublished data) from these two strains confirmed that this predicted a *cryIII*-type gene is novel. The 1.6-kb PCR-amplified fragment of *B. thuringiensis* subsp. *indiana* was not restricted by *Pst*I and *Xba*I (Fig. 4). However, the partial nucleotide sequence of this PCR fragment (unpublished data) revealed that the sequence was similar to that of the *cryIII*-type gene. The PCR amplification patterns of all other tested *B. thuringiensis* strains (Fig. 2 and Table 3) were complicated. Therefore, the *cry*-type genes of these strains were not identified.

**Identification of *cryIC*-, *cryIC(b)*-, *cryID*-, *cryIE*-, and *cryIF*-type genes from *B. thuringiensis* strains.** The restriction pattern of the 1.6- to 1.7-kb PCR-1 fragment (Table 2) cannot distinguish the *cryIC(b)*, *cryID*, and *cryIF* genes since the sizes of the major specific restriction fragments from these *cry*-type genes are similar (Table 2). To solve this problem, another pair of universal oligonucleotide primers, K5un3 and K3un3 (Table 1), were designed to amplify the *cryIC*, *cryIC(b)*, *cryIE*, *cryID*, and *cryIF* genes specifically. By use of this pair of oligonucleotide primers, only *B. thuringiensis* subspecies *thuringiensis*, *kenyae*, *galleriae*, *morrisoni*, *wuhanensis*, and *aizawai* produced a 1.4-kb PCR-2 product (Fig. 3A). The PCR-2 fragment was restricted with *Eco*RI and *Pst*I; the *cry*-type genes were then

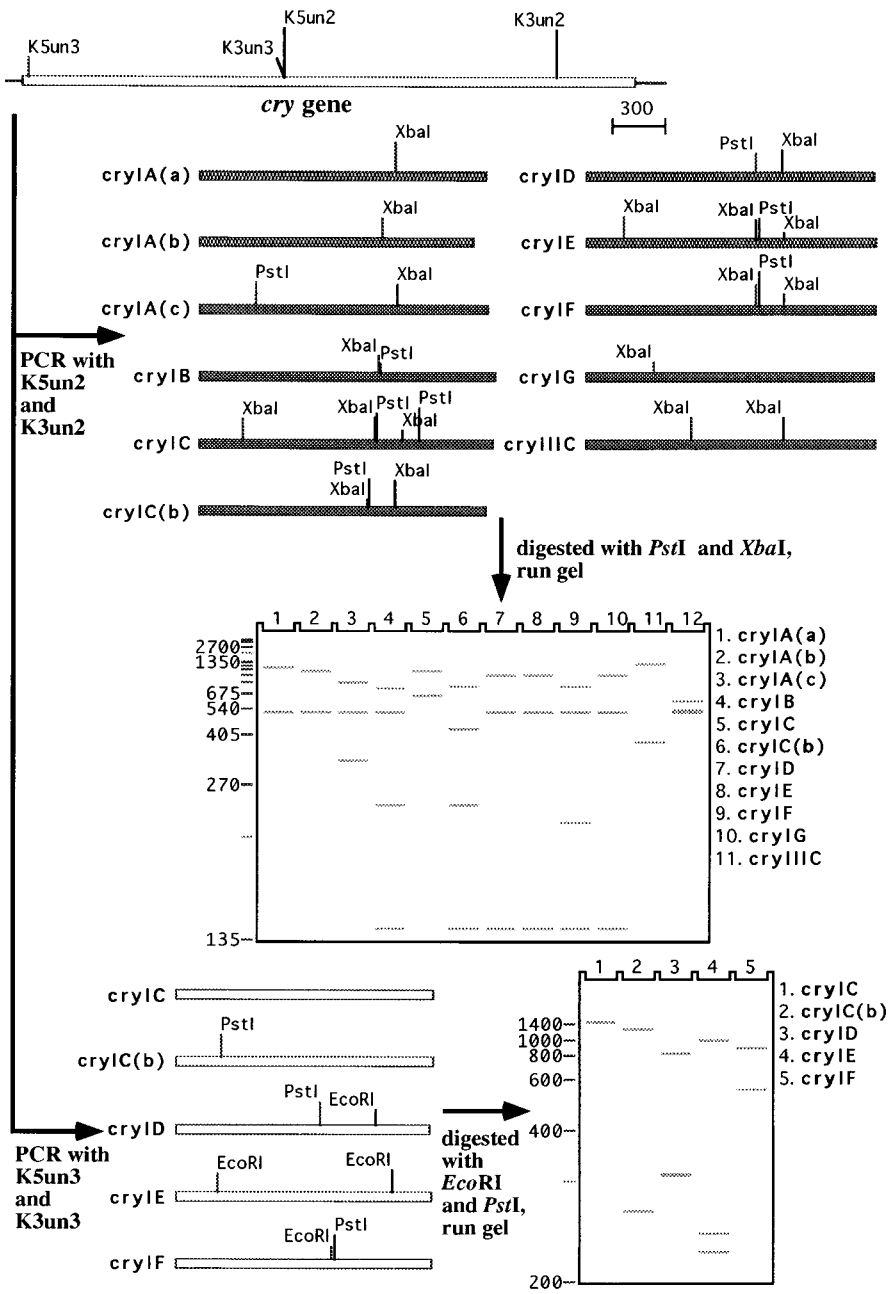


FIG. 1. Strategy for the analysis of the *cry* genes from *B. thuringiensis* strains on the basis of RFLP of the PCR-amplified DNA. The relative positions of the oligonucleotide primers priming the DNA template are indicated. K5un3-K3un3 and K5un2-K3un2 are the two oligonucleotide primer pairs for PCR amplification. The restriction map of the distinct *cry*-type genes is shown. The computer-predicted RFLP patterns of the *cry*-type genes analyzed by agarose gel electrophoresis are also shown.

determined by the analysis of their RFLP patterns (Table 2). The restriction patterns of these 1.4-kb PCR-2 fragments are shown in Fig. 3B. The results of *cry* gene typing of these *B. thuringiensis* strains are listed in Table 3. This PCR-RFLP method confirmed that *B. thuringiensis* subsp. *kenyae* also contained a *cryIC(b)* gene, that *B. thuringiensis* subspecies *galleriae*, *morrisoni* HD-12, and *wuhanensis* each also contained a *cryIC(b)* and a *cryIF* gene, and that *B. thuringiensis* subsp. *aizawai* contained a *cryIC* and a *cryID* gene. The predicted *Eco*RI-*Pst*I restriction pattern of the *cryIF* gene consists of a 879- and a 548-bp fragment (Table 2). The restriction pattern of the 1.4-kb product from *B. thuringiensis* subsp. *morrisoni* produced these two major fragments (Fig. 3B). However, the restriction pattern of the 1.4-kb product from *B. thuringiensis* subsp. *galleriae* produced a 650- and a 540-bp fragment (Fig. 3B). The similarity of the RFLP patterns indicated that this strain may contain a novel *cryIF*-type gene. The compiled results of the *cry*-type genes of all tested *B. thuringiensis* strains by use of these two PCR-RFLP *cry* gene typing methods are summarized in Table 3.

**Cloning of a novel *cryI*-type gene from a strain of *B. thuringiensis* subsp. *wuhanensis*.** Eleven distinct PCR products were

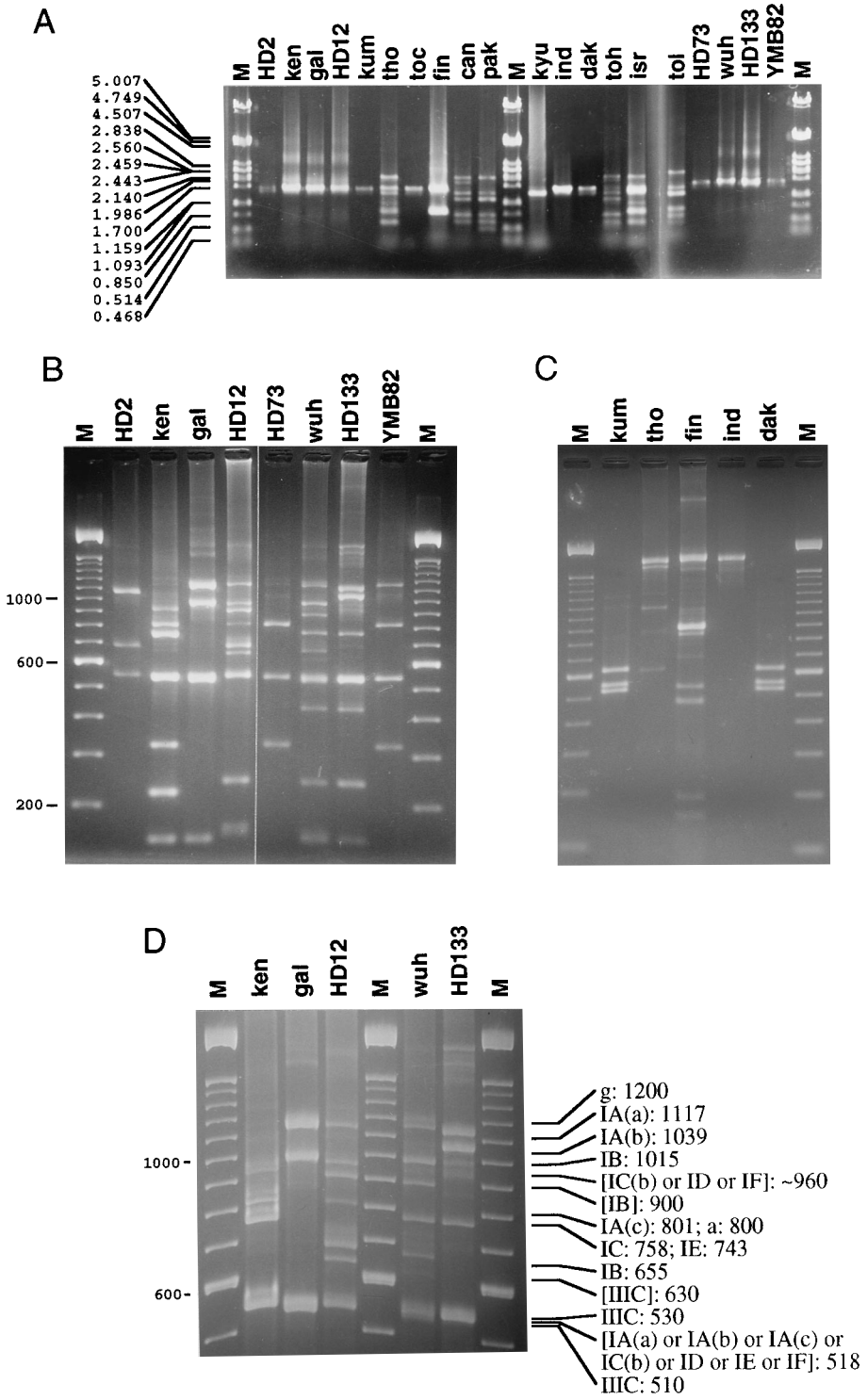


FIG. 2. PCR amplification with oligonucleotide primers K5un2 and K3un2 and RFLP patterns of the distinct *cry*-type genes. (A) PCR amplifications of the DNAs from various *B. thuringiensis* strains are shown. Most of the templates produced a fragment of around 1.6 kb after PCR amplification. (B to D) The PCR-amplified fragments were restricted with *Pst*I and *Xba*I, and the RFLP patterns of the corresponding *cry* genes were analyzed by 3% agarose gel electrophoresis. (D) The restriction patterns of the PCR products from *B. thuringiensis* subspecies *kenyae*, *galleriae*, *morrisoni* HD-12, *wuhanensis*, and *aizawai* HD-133 were complicated. To resolve restriction fragments ranging from 500 to 1200 bp, a long-run agarose gel electrophoresis was performed. The prospective *cry*-type genes and the typical sizes (in base pairs) of the restriction fragments of the corresponding PCR products are indicated to the right of the gel. Uncertain *cry*-type genes are shown in brackets. g: 1200 is an example of an unidentified restriction fragment. Lanes M, 100-bp-sized markers.

TABLE 3. Compiled results of *cry*-type gene combinations of various *B. thuringiensis* strains

| *B. thuringiensis* subspecies and strain | PCR-1–RFLP[a,b] | PCR-2–RFLP[b,c] | Compiled *cry*-type gene[a] |
|---|---|---|---|
| *thuringiensis* HD-2 | [IA(a)], 1,060, 518; [IB], 1,060, 655 | [IA(a) or IA(b)], 726, 493 | IA(b), IB |
| *kenyae* HD-5 | [IC(b) or ID or IF], 960, 518, 140; IA(c), 801, 518, 322; IE, 743, 518, 218, 140 | IC(b), 1,196, 261; IE, 986, 238, 221; IA(c), 726, 434, 244 | IA(c), IC(b), IE |
| *galleriae* HD-29 | IA(a), 1,120, 518; [IC(b) or ID or IF], 960, 518, 140 | IC, 1,457; IC(b), 1,196, 261; [IF], 650, 540, 229 | IA(a), IC†, IC(b), IF† |
| *morrisoni* HD-12 | IA(a), 1,120, 518; [IC(b) or ID or IF], 960, 518, 140; [IB], 900, 655; a, 800; [IIIC], 620, 530, 510; IC, 239; b, 150; c, 160 | IC, 1,457; IC(b), 1,196, 261; IF, 879, 540; [IA(a) or IA(b)], 726, 493 | IA(a), IB†, IC†, IC(b), IF, III† |
| *kumamotoensis* HD-867 | [IIIC], 620, 530, 510 | No product | III† |
| *thompsoni* HD-542 | NI[e] | No product | NI |
| *tochigiensis* HD-868 | d, 1,680 | No product | III† |
| *finitimus* HD-3 | e, 1,680 | No product | III† |
| *canadensis* HD-224 | NI | No product | NI |
| *pakistani* HD-395 | NI | No product | NI |
| *kyushuensis* HD-541 | NI | No product | NI |
| *indiana* HD-521 | f, 1,680 | No product | III† |
| *dakota* HD-932 | [IIIC], 620, 530, 510 | No product | III |
| *tohokuensis* HD-866 | NI | No product | NI |
| *israelensis* HD-567 | NI | No product | NI |
| *tolworthi* HD-537 | NI | No product | NI |
| *kurstaki* HD-73 | IA(c), 801, 518, 322 | No product | IA(c) |
| *wuhanensis* HD-525 | g, 1200; [IA(a)], 1,150, 518; [IA(b)], 1,000, 518; [IC(b) or ID or IF], 960, 518, 140; [IB], 900, 655; IC, 758, 423, 239, 140; [IIIC], 620, 530, 510 | IC, 1,457; IC(b), 1,196, 261; IF, 879, 548 | IA(a)†, IA(b)†, IB†, IC†, IC(b), IF, III† |
| *aizawai* HD-133 | IA(a), 1,120, 518; IA(b), 1,050, 518; [IA(b)], 1,000, 518; [IC(b) or ID or IF], 960, 518, 140; IC, 758, 423, 239, 140 | IC, 1,457; ID, 819, 310, 307; [IA(a) or IA(b)], 726, 493, 244 | IA(a), IA(b), IC, ID |
| YMB82 | IA(a), 1,140, 518; IA(c), 801, 518, 322 | No product | IA(a), IA(c) |

[a] PCR with K5un2 and K3un2 followed by *Pst*I and *Xba*I digestion.
[b] The prospective *cry*-type gene and the sizes (in base pairs) of the restriction fragments of the corresponding PCR products are indicated. Uncertain *cry*-type genes are shown in brackets. The letters a to g preceding a comma and not part of a gene designation indicate unidentified restriction fragments or the unrestricted PCR fragments.
[c] PCR with K5un3 and K3un3 followed by *Eco*RI and *Pst*I digestion.
[d] †, novel *cry*-type gene determined by restriction mapping or DNA sequences.
[e] NI, not identified.

cloned into pUC18 for further studies (unpublished data). Seven clones were from *B. thuringiensis* subsp. *wuhanensis*, two were from *B. thuringiensis* subsp. *israelensis*, one was from *B. thuringiensis* subsp. *kumamotoensis*, and one was from *B. thuringiensis* subsp. *indiana*. Endonuclease restriction analysis of these clones showed that the restriction maps of some PCR products were similar to that of the published restriction map of the corresponding *cry* gene (unpublished data). In contrast, the endonuclease restriction analysis of the cloned PCR products reveal that *B. thuringiensis* subsp. *wuhanensis* may contain a novel *cryIA(a)* and *cryIB* gene (Fig. 4).

The restriction map of the PCR product PCRt29 is not identical to that of the predicted *cryIA(a)* gene (Fig. 4A). To prove that this PCR clone is derived from either plasmid or chromosomal DNA of *B. thuringiensis* subsp. *wuhanensis*, and also is a novel *cry* gene, we used PCRt29 to probe a *cry* gene from the genomic library of *B. thuringiensis* subsp. *wuhanensis*. Southern hybridization results indicated that 2.6-, 5.0-, 5.3-, 10-, and 20-kb *Hin*dII fragments of the restricted total DNA hybridized with the probe (data not shown). The 5.3-kb *Hin*dII fragment was cloned. Results of restriction analysis indicated that PCRt29 and the 5.3-kb clone are identical (Fig. 4B). The DNA sequence of this 5.3-kb clone has been completed (unpublished data). Nucleotide sequence analysis of this clone indicated that the five conserved blocks (21) of a *cry* gene were found in the 5.3-kb clone. By use of the Pileup program in the Genetics Computer Group Sequence Analysis Software Package to analyze the position of this *cry* gene in the evolutionary dendrogram of *cry* genes, we found that the *cry* gene had only 43 to 70% homology with all known *cry* gene sequences. Thereby, we concluded that this *cry* gene from *B. thuringiensis* subsp. *wuhanensis* must be a novel *cry*-type gene.

## DISCUSSION

Multiplex PCR is becoming an increasingly important method to identify the existence of specific *cry*-type genes in *B. thuringiensis* strains. However, the major limitation of this method is that the existence of a novel *cry* gene from a *B. thuringiensis* strain cannot be identified easily. Here we provide a complementary, facile method to detect novel *cry* genes in *B. thuringiensis* strains. Some *B. thuringiensis* strains such as *B. thuringiensis* subsp. *wuhanensis* exhibit insecticidal activities, but the *cry*-type gene of such strains has not been identified (36); therefore, some novel *cry* genes may be present in these *B. thuringiensis* strains. To understand whether there are some novel *cry*-type genes in these *B. thuringiensis* strains, two pairs of universal oligonucleotide primers were designed for PCR

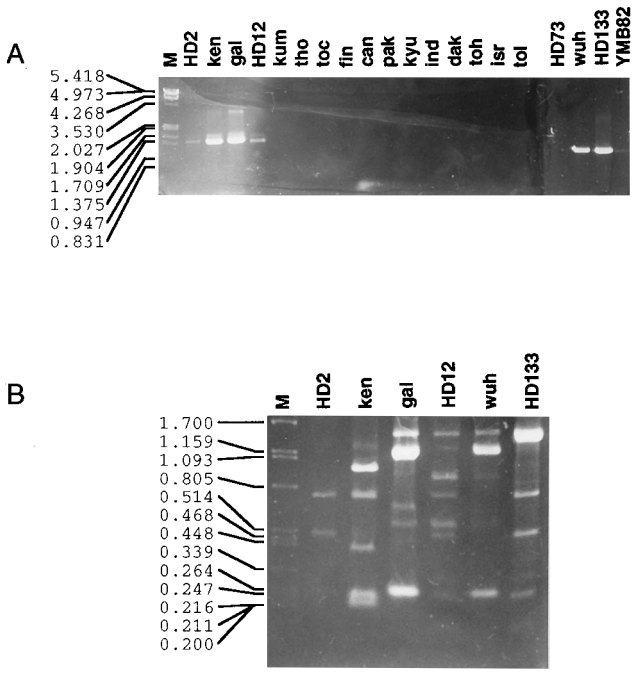


FIG. 3. PCR amplification with oligonucleotide primers K5un3 and K3un3 and RFLP patterns of the distinct *cry*-type genes. (A) PCR amplifications of the DNAs from various *B. thuringiensis* strains are shown. Only six DNA templates from various *B. thuringiensis* strains produced fragments of around 1.4 kb. (B) The PCR-amplified products were then restricted with *Eco*RI and *Pst*I, and the RFLP patterns of the corresponding *cry* genes were analyzed by 3% agarose gel electrophoresis. Lanes M, molecular size markers.

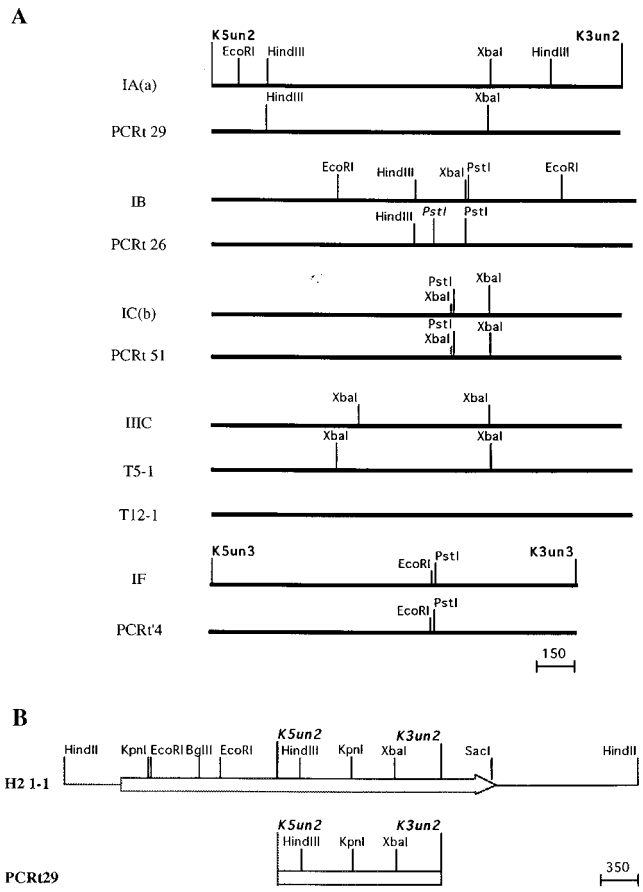


FIG. 4. Restriction mapping of the PCR fragments (A) and the genomic clone (B) from *B. thuringiensis* strains. The PCR fragments were cloned into pUC18. The similarities of the restriction map of the PCR clones with those of the corresponding known *cry* genes are shown. It is noted that clones PCRt29, PCRt26, PCRt51, and PCRt′4 were from *B. thuringiensis* subsp. *wuhanensis*, T5-1 was from *B. thuringiensis* subsp. *kumamotoensis*, and T12-1 was from *B. thuringiensis* subsp. *indiana* (no *Xba*I and *Pst*I sites). The oligonucleotide primer sets K5un2-K3un2 and K5un3-K3un3, used to amplify the 1.6-kb PCR-1 and the 1.4-kb PCR-2 products, are also shown. The restriction map of the 5.3-kb *Hin*dII fragment from the genomic library of *B. thuringiensis* subsp. *wuhanensis* is shown in panel B. For details, see Results.

amplification instead of using multi-oligonucleotide primer pairs in multiplex PCR amplification.

The nucleotide sequences of oligonucleotide primers K5un2 and K3un2 are highly conserved with most published *cryI*-type gene sequences (Table 1). Presumably, these sequences may be present in other unknown *cry* genes as well. In contrast, most of the oligonucleotide primers designed for multiplex PCR amplification were situated in the hypervariable regions of the *cry* genes so that these oligonucleotide primers can be used only to amplify the templates of the closely related *cry* gene sequences. Therefore, this specific homologous relationship between oligonucleotide primers and the template DNAs in the multiplex PCR amplification systems would largely reduce the chance to detect the presence of novel *cry* gene sequences from the tested *B. thuringiensis* strains.

The 1.6-kb unique PCR fragments amplified by oligonucleotide primers K5un2 and K3un2 with template DNAs from any distinct *B. thuringiensis* strain may therefore include all possible *cryI*-type genes that can be easily identified by RFLP pattern analysis (Table 2 and Fig. 2) of the PCR products. If any unexpected RFLP pattern is detected, this result may indicate the presence of a prospective novel *cry* gene sequence. Oligonucleotide primers K5un3 and K3un3 were designed specifically for the amplification of *cryIC*, *cryIC(b)*, *cryID*, *cryIE*, and *cryIF* gene sequences (Table 2 and Fig. 3). A combination of these two PCR-RFLP results enabled us to identify 14 distinct *cry*-type genes from 20 tested *B. thuringiensis* strains (Table 3). Those *cry*-type genes included *cryIA(a)*-, *cryIA(a)*†-, *cryIA(b)*-, *cryIA(b)*†-, *cryIA(c)*-, *cryIB*†-, *cryIC*-, *cryIC*†-, *cryIC(b)*-, *cryID*-, *cryIE*-, *cryIF*-, *cryIF*†-, and *cryIII*†-type genes. Among them, the sequences of *cryIA(a)*†-, *cryIA(b)*†-, *cryIB*†-, *cryIC*†-, *cryIF*†-, and *cryIII*†-type genes were found to be different from the corresponding published *cry* gene sequences by either their restriction patterns (Fig. 4) or partial nucleotide sequences of the PCR products (unpublished data). This PCR-RFLP method is effective in detecting the existence of *cryI*-type genes. In addition, by use of this method, we were also able to detect the existence of a *cryIII*-type gene in *B. thuringiensis* subspecies *kumamotoensis*, *dakota*, and *indiana.*

Detection of a *cry*-type gene combination by use of this PCR-RFLP method in some well-studied *B. thuringiensis* strains such as *B. thuringiensis* subspecies *thuringiensis* HD-2, *kurstaki* HD-73, and *aizawai* HD-133 and isolate YMB-82 completely agreed with results of previous studies (11, 36). In addition to *cryIA(c)* and *cryIE* genes (36), we detected one more *cryIC(b)* gene in *B. thuringiensis* subsp. *kenyae*. The most controversial data were those of the *cry*-type gene combination of *B. thuringiensis* subsp. *galleriae*. From the published data (36), this subspecies harbors *cryID*, *cryIG*, and *cryIIIC* genes. In contrast, only *cryIA(a)*, *cryIC*, *cryIC(b)*, and *cryIF*†-type genes were detected by this method. Kalman et al. (23) found that *B. thuringiensis* subsp. *galleriae* contains *cryIA(b)*, *cryIA(c)*, *cryIC(b)*, and *cryID* genes. We do not know why the *cry*-type gene

combinations of this strain are so diversified. This is probably due to the differences of the strains used in different laboratories.

The *cry*-type gene combinations of *B. thuringiensis* subspecies *morrisoni* HD-12 and *wuhanensis* have not been reported previously. *B. thuringiensis* subspecies *morrisoni* HD-12 (19) and *wuhanensis* (unpublished data) were reported to have a wide range of toxicity against target insects. Probably, there must be some novel *cry*-type genes existing in these strains. Interestingly, five *cry*-type genes [*cryIA(a)*-, *cryIB*-, *cryIC*-, *cryIC(b)*-, and *cryIF*-type genes] in *B. thuringiensis* subsp. *morrisoni* HD-12 and six *cry*-type genes [*cryIA(a)*†-, *cryIA(b)*†-, *cryIB*†-, *cryIC*-†, *cryIC(b)*-, and *cryIF*-type genes] in *B. thuringiensis* subsp. *wuhanensis* were detected by this *cry* gene typing method. A *cryI*-type (Fig. 4B) gene and a *cryIC*-type (unpublished data) gene from the genomic library of *B. thuringiensis* subsp. *wuhanensis* were cloned. The completed nucleotide sequence (unpublished data) of the *cryI*-type gene confirmed that it had only 40 to 70% homology with the corresponding published *cry*-type gene. Therefore, this must be a novel *cryI*-type gene. Analysis of a dendrogram showed that this novel *cry* gene may be positioned between *cryIF* and *cryIB* genes.

In addition to the 1.6-kb fragment, some strains produced extra PCR products ranging from 2.0 to 1.0 kb. This might be due to nonspecific priming of the oligonucleotide primers. Perhaps the size of an unknown *cry* gene amplified from the typing method is different from that of the predicted size of the known *cry* gene. Brown and Whiteley (6) reported that two or three *cry* genes may be positioned next to each other, forming an operon. If this is the case, priming of an oligonucleotide primer to the neighbor *cry* gene may produce a PCR product larger than 1.4 or 1.6 kb. Since we analyzed only the 1.4- and 1.6-kb specific amplified fragments, artifacts caused by the amplification of flanking *cry* genes would not have occurred in this study.

Nonspecific amplification is one of the major limitations of PCR. To reduce this limitation as much as possible, precautionary procedures were taken during the entire proceedings of this work. First, we analyzed only the specific 1.4- and 1.6-kb PCR fragments amplified by the two specific oligonucleotide primer sets. Second, the predicted *cry*-type gene combinations of the *B. thuringiensis* strains listed in Table 3 were required to agree with data from both PCR-1–RFLP and PCR-2–RFLP. Restriction maps of some PCR clones such as PCRt51 and PCRt′4 (Fig. 4A) were found to be identical to the published data, indicating that this PCR-RFLP method really can identify known *cry* gene sequences. However, restriction maps of many PCR clones, e.g., from *B. thuringiensis* subsp. *wuhanensis*, were found to be similar but not identical to the published data (Fig. 4A). To confirm whether these clones are novel *cry* genes, we selected a clone, PCRt29, to probe the *cry* gene(s) from the genomic library of *B. thuringiensis* subsp. *wuhanensis*. The cloning results confirmed that the *cry* gene cloned from the genomic library is indeed a novel *cry* gene (Fig. 4B). In conclusion, the PCR-RFLP is a reasonably reliable method to identify novel *cry* genes.

In addition to detecting novel *cry* genes, these two universal oligonucleotide primer sets used for the PCR-RLFP typing method may also be applied to detect the organization of the *cry* genes on the plasmid. Since the PCR amplification system produces only a specifically sized product, it is possible to use this system to detect the copy number of the *cry* genes existing in a *B. thuringiensis* strain. Furthermore, this amplification system may also be used to measure quantitatively the differential *cry* gene expression at the transcriptional level via reverse transcription-PCR.

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