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# Determination and Distribution of *cry*-Type Genes of *Bacillus thuringiensis* Isolates from Taiwan

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**Using PCR with a set of specific oligonucleotide primers to detect *cryI*-type genes, we were able to screen the *cry*-type genes of 225 *Bacillus thuringiensis* soil isolates from Taiwan without much cost in time or labor. Some combinations of *cry* genes (the *cry*-type profile) in a single isolate were unique. We identified five distinct profiles of crystal genes from the *B. thuringiensis* soil isolates from Taiwan. The *cry* genes included *cryIA(a)*, *cryIA(b)*, *cryIA(c)*, *cryIC*, *cryID*, and *cryIV*. Interestingly, 501 *B. thuringiensis* isolates (93.5% of the total number that we identified) were isolated from areas at high altitudes. The profiles of *cry*-type genes were distinct in all isolation areas. The distribution of *cry*-type genes of our isolates therefore depended on geography. Using PCR footprinting to detect *cryIC*-type genes, we identified two distinct *cryIC* footprints from some of our isolates, indicating that these isolates may contain novel *cryIC*-type genes. *B. thuringiensis* isolates containing *cryIA(a)*-, *cryIA(b)*-, and *cryIA(c)*-type genes exhibited much greater activity against *Plutella xylostella* than did other isolates, indicating that multiple *cry*-type genes may be used as markers for the prediction of insecticidal activities.**

*Bacillus thuringiensis* is a spore-forming gram-positive bacterium. During sporulation, the intracellular insecticidal crystal proteins (Cry proteins) are produced as phase-bright inclusions (4). These proteins are toxic to insect larvae in the orders *Lepidoptera*, *Diptera*, and *Coleoptera* (5, 15). The Cry protein from *B. thuringiensis* has been developed as a successful biological agent to control insect pests (1).

The insecticidal crystal protein genes (*cry* genes) are normally associated with plasmids with high molecular masses (9). 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 (11).

*B. thuringiensis* soil isolates are distributed globally (7, 13, 17, 19). To obtain novel *B. thuringiensis* strains for the production of Cry proteins, isolation of numerous new *B. thuringiensis* strains is becoming a routine activity in many industries. *B. thuringiensis* strains are classified into 34 serovars (8). However, the characterization of so many isolates by serotyping requires much labor and time. Because serotypes of *B. thuringiensis* strains do not directly reflect the specific *cry* gene classes in the strains, prediction of insecticidal activity of a *B. thuringiensis* strain on the basis of serotyping seems impractical. By using PCR, we were able to screen the *cry*-type genes of 225 *B. thuringiensis* isolates from Taiwan without much cost in labor or time; thus, the determination of *cry*-type genes by the PCR product profile of the specific *cry*-type genes from *B. thuringiensis* strains is a feasible way to characterize numerous *B. thuringiensis* isolates. Our results indicate that some *B. thuringiensis* isolates may contain novel *cryIC* genes.

## MATERIALS AND METHODS

**Bacterial strains and media.** *B. thuringiensis* strains, other than those isolated from Taiwan, were kindly provided by the *Bacillus* Genetic Stock Center, Ohio State University, Columbus. Nutrient broth and nutrient agar were from Difco. L broth and L agar were as previously described (18). CYS medium was prepared as described by Yamamoto (24). SA agar contains, per liter, Spizizen salt (100 ml, 5×) (20), Casamino Acids (25 ml, 20%), glucose (12.5 ml, 20%), and $MnSO_4$ (0.5 ml, 5 mM). Cultures of *B. thuringiensis* strains were grown at 30°C with vigorous shaking in L broth or CYS medium for 48 h.

**Soil collection.** Twenty-one soil samples were collected from five selected regions in Taiwan. In Taipei (northern part of Taiwan), seven samples ($TP_I$ to $TP_{VII}$) were collected from areas of low altitude, and two samples (TPYMI and $TPYM_{II}$) were collected from the Yang Ming Mountain area, which is several hundred meters above sea level. In Taichung (central Taiwan), six samples ($TC_I$ to $TC_{VI}$) were collected from low-altitude areas. In the central mountain range (at least 3,000 meters above sea level), four samples ($YS_I$ to $YS_{IV}$) were collected from Yu Shan, and two samples ($SS_I$ and $SS_{II}$) were collected from Shieh Shan. Soil samples were collected from the surface to up to 2 in. (ca. 5 cm) below the surface. Each sample was placed in a standard-sized sampling vial (20 ml) and covered with clear cheesecloth instead of a screw cap because of concerns that a plastic bag could suffocate *B. thuringiensis* strains that were in the vegetative stage (17). All samples were then kept in 25°C in an air-conditioned room.

**Screening for *B. thuringiensis* soil isolates.** Soil samples were placed in an air-conditioned room for a few days in order to equilibrate the moisture in the soil samples. Various soil samples (one tablespoonful, weighing about 1 g) were suspended in nutrient broth (20 ml) in a test tube (50 ml). The samples were heated to 55°C for 10 min to remove nonsporeformers in the soil. The samples were diluted $10^4$-fold, and then each diluted sample (200 μl) was spread on a nutrient agar plate. The plates were dried and incubated at 30°C for at least 3 days. Ten single colonies with a morphology similar to that of *B. thuringiensis* were randomly selected from each of the plates and examined by phase-contrast microscopy. Eventually,

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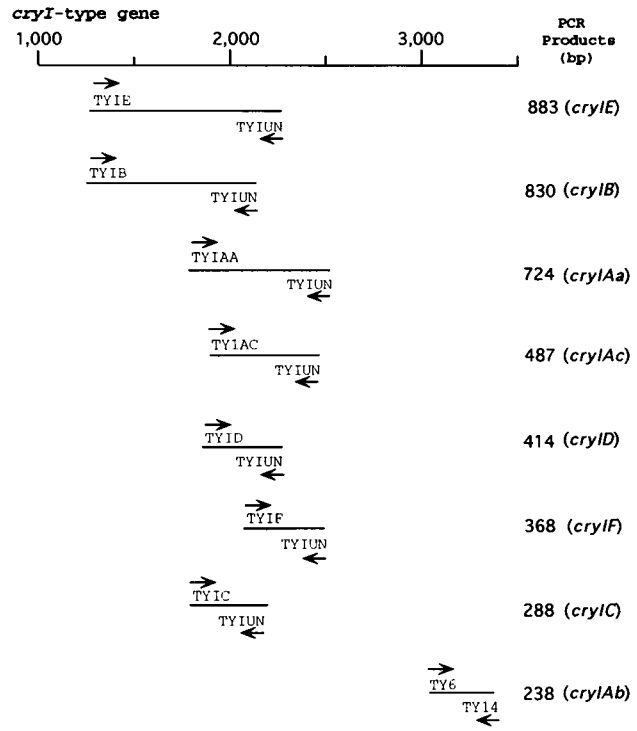


FIG. 1. Strategy for detection of *cryI*-type genes. Positions of eight pairs of PCR oligonucleotide primers relative to those of the corresponding *cry* genes for amplification of specific *cryI*-type gene fragments are shown. Arrows pointing to the right indicate the locations of forward oligonucleotide primers, whereas arrows pointing to the left indicate locations of reverse oligonucleotide primers. Sizes of specific amplified PCR fragments are indicated. The sequences of all oligoprimers used in this work were previously described (16).

only bacteria that produced spores and phase-bright inclusions were retained for further examination.

**Extraction of total DNA from *B. thuringiensis* strains for PCR analysis.** A freshly (overnight) isolated colony incubated at 30°C on either a nutrient agar or an L agar plate was selected and restreaked on an SA plate. The plate was incubated at 37°C for 4 to 6 h. Cells (two loopfuls) from the SA 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 subsequent DNA extraction was as described by Birnboim (2). Finally, the washed DNA pellets were resuspended in 20 μl of 1× TE (10 mM Tris-HCl [pH 8], 1 mM EDTA).

**PCR.** The DNA solution prepared was diluted 50-fold before being used for PCR analysis. To identify *cryI*-type genes, we used eight forward oligonucleotide primers and two reverse oligonucleotide primers (14) (Fig. 1) together in one reaction. Oligonucleotide primer TYIUN (Fig. 1) is a universal reverse primer that binds to the specific sequences of various *cryI*-type genes [except the *cryIA(b)*-type gene]. However, when the nucleotide sequences of the *cryI*-type genes align with the 5′ end, the positions of the specific sequences for the universal primer TYIUN from distinct *cryI*-type genes become misaligned. To detect *cryIC*-type genes, we synthesized six oligonucleotide primer pairs (14) (see Fig. 5) based on the *cryIC* gene sequence (12) and used them together in one PCR.

For PCR amplification, the diluted template DNA (0.5 μl) was mixed with PCR mixture (9.5 μl, 1×; 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% [wt/vol] gelatin) containing deoxynucleoside triphosphates (0.2 mM each), mixed oligonucleotide primers (0.2 mM each), and AmpliTaq polymerase (0.25 U) (Perkin-Elmer Cetus). PCR was performed for 25 cycles with denaturation of template DNA at 94°C for 1 min, annealing templates and oligonucleotide primers at 52°C for 2 min, and extension of PCR products at 72°C for 3 min. The PCR products were separated and analyzed on a 2% agarose gel.

TABLE 1. Distribution of *B. thuringiensis* soil isolates from selected regions of Taiwan

| Region and designation | No. (%) of isolates | | |
|---|---|---|---|
| | Total | Sporeformers | *B. thuringiensis* |
| Taipei (TP) | 555 | 164 | 6 (3.7) |
| Taipei, Yang Ming (TPYM) | 200 | 148 | 104 (70.3) |
| Taichung (TC) | 414 | 146 | 29 (19.9) |
| Yu Shan (YS) | 243 | 212 | 193 (91.0) |
| Shieh Shan (SS) | 255 | 255 | 204 (80.0) |
| Total | 1,667 | 925 | 536 |

**Preparation of *B. thuringiensis* cell extracts for bioassay.** *B. thuringiensis* strains were grown in CYS medium at 30°C with vigorous shaking (300 rpm) for up to 3 days or until spores and crystals were released. Spores and crystals were harvested and washed at least three times with NaCl (0.5 M) solution containing Tris-HCl (10 mM) (pH 8) and EDTA (2 mM). Finally, the washed pellets were resuspended in a volume of TE equivalent to that of the original culture.

**Other biochemical techniques.** Agarose gel electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described by Maniatis et al. (16) and Hames (10), respectively. Western blotting (immunoblotting) was performed according to the method of Towbin et al. (21). Anti-CryI antiserum was raised against Cry proteins from *B. thuringiensis* subsp. *aizawai* HD133.

**Bioassay.** The bioassay was performed with three locally collected and laboratory-reared species, *Plutella xylostella*, *Heliothis armigera*, and *Spodoptera litura*. Samples to be assayed were mixed with an appropriate diet and fed to third-instar larvae at 25°C. For primary screening tests, *B. thuringiensis* cell extracts (10,000 ppm) were used to establish the efficacy of each insect species tested. Samples with sufficient activity were then evaluated in a dose-response test. For both tests, cell extracts from *B. thuringiensis* subsp. *kurstaki* HD1 were used as a positive control. The concentrations of cell extracts were expressed as parts per millien of diet. The mortality rates for 20 insect larvae tested were recorded after incubation for 3 to 4 days at 25°C. The data for 50% lethal concentrations, which were determined by probit analysis, were the averages from three experiments.

## RESULTS

**Isolation and characterization of *B. thuringiensis* soil isolates.** After heat treatment, soil samples were diluted and plated on nutrient agar plates. The distribution and number of the *B. thuringiensis* isolates from the soil samples are shown in Table 1. Of 1,667 isolates that we examined, 536 produced both spores and phase-bright inclusions. Surprisingly, most of the *B. thuringiensis* isolates (501 [93.5%]) were isolated from areas at high altitudes, whereas only 35 isolates (6.5%) were from areas at low altitudes.

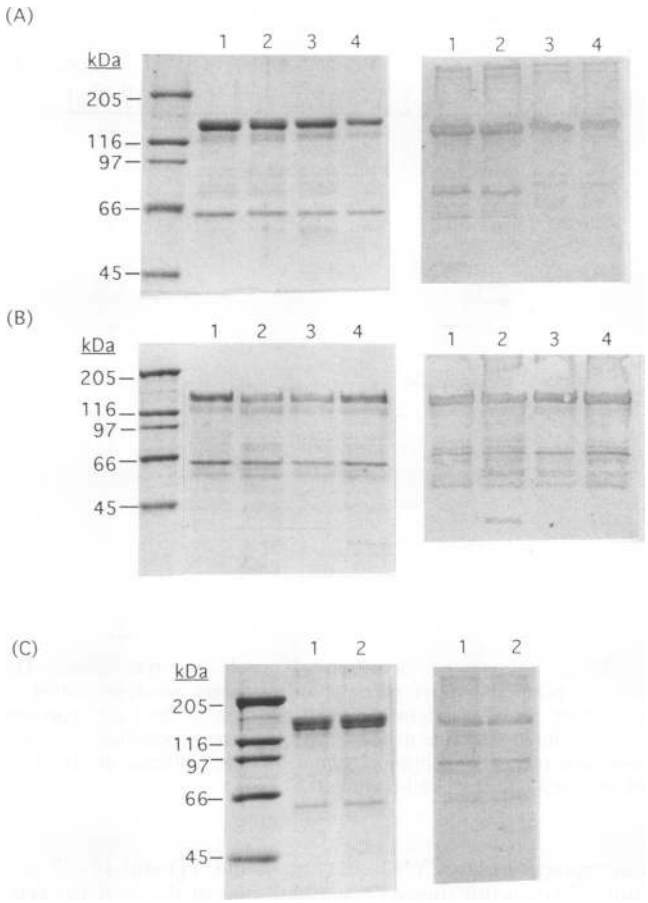


FIG. 2. Cry protein profiles of *B. thuringiensis* isolates. SDS–7.5% PAGE of cell extracts from *B. thuringiensis* isolates (left panels) and immunoblotting of an identical gel (right panels) are shown. (A) Samples with one protein band in the 130- to 140-kDa region. Lanes 1 to 4, extracts from isolates TC-16 to TC-19, respectively. (B) Samples with two protein bands in the 130- to 140-kDa region. Lanes 1 to 4, extracts from isolates YS-85 to YS-88, respectively. (C) Samples with three protein bands in the 130- to 140-kDa region. Lanes 1 and 2, extracts from isolates TC-22 and TC-23, respectively. The unlabeled lanes contain molecular mass markers.

All *B. thuringiensis* isolates were further characterized by SDS-PAGE and Western blotting of the protein products produced from the isolates. We observed three typical protein profiles around the 130- to 140-kDa area with most isolates. Some isolates produced one, some produced two, and some produced three proteins with bands in this vicinity. Selected samples are shown in Fig. 2. All protein bands in this area from the *B. thuringiensis* isolates reacted with antiserum raised against crystal protein from *B. thuringiensis* subsp. *aizawai* HD133 (Fig. 2), which contains *cryIA*(*a*)-, *cryIA*(*b*)-, *cryIC*-, and *cryID*-type genes (Fig. 3).

The protein profiles of four isolates, TPYM-26, -52 -66, and -91, were distinct from those of all other *B. thuringiensis* isolates. They produced proteins of 130, 65, 40, and 27 kDa (7). This protein profile resembled that of *B. thuringiensis* subsp. *israelensis* (23). This likeness was confirmed by plasmid profiles (data not shown) and by PCR amplification profiles.

**Determination of *cry*-type genes of *B. thuringiensis* isolates by PCR amplification.** Kalman et al. (14) designed eight specific forward and two reverse oligonucleotide primers to amplify specific DNA fragments from eight *cryI*-type genes [*cryIA*(*a*), *cryIA*(*b*), *cryIA*(*c*), *cryIB*, *cryIC*, *cryID*, *cryIE*, and *cryIF*]. The predicted sizes of the PCR-amplified products and the relative positions of the specific oligonucleotide primers with respect to the corresponding *cry* genes are shown in Fig. 1. The reliability of the mixed *cryI*-type oligonucleotide primers for detection of *cry*-type genes from *B. thuringiensis* strains was verified (Fig. 3) by use of well-defined *B. thuringiensis* strains. The *cry*-type genes identified in these *B. thuringiensis* strains by the PCR amplification method agreed with published data (25).

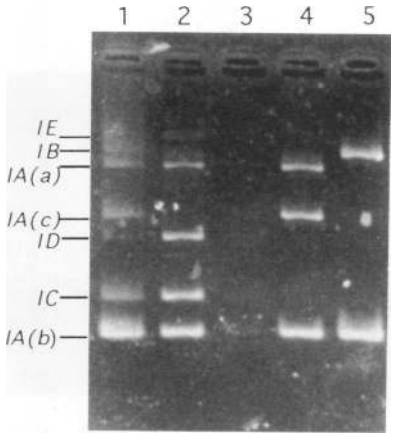


FIG. 3. PCR amplification profiles of *cryI*-type genes of some *B. thuringiensis* strains. Specific PCR-amplified *cryI*-type gene fragments of *B. thuringiensis* strains obtained by using the mixed *cryI*-type oligoprimers were run on a 2% agarose gel. Lane 1, PCR-amplified *cryI*-type gene fragments from mixed DNAs of eight *cryI*-type genes. The positions of specific *cry*-type gene fragments are indicated. Lanes 2 to 5, amplifications of DNA from *B. thuringiensis* subsp. *aizawai* HD133, *B. thuringiensis* subsp. *israelensis*, *B. thuringiensis* subsp. *kurstaki* HD1, and *B. thuringiensis* subsp. *thuringiensis*, respectively.

In conjunction with tests of toxicity against three locally reared insect pests (see Tables 5 and 6), we selected 225 *B. thuringiensis* isolates (Table 2) to determine *cry*-type genes of the respective strains. Five distinct *cry*-type gene profiles (Table 2) were detected by PCR. Selected PCR amplification results are shown in Fig. 4. Using mixed oligonucleotide primers (6) for PCR to detect *cryIII*- and *cryIV*-type genes from *B. thuringiensis* isolates, we found that only four isolates from Yang Ming Mountain (TPYM-5, -26, -66, and -91) contained a *cryIV*-type gene. We detected no *cryIII*-type gene in our *B. thuringiensis* isolates.

Isolates from each geographic location had a characteristic

TABLE 2. Distribution of *cry*-type gene profiles of *B. thuringiensis* isolates from Taiwan

| Location | No. of *B. thuringiensis* isolates with the following *cry*-type gene(s): | | | | |
|---|---|---|---|---|---|
| | *cryIA*(*a*), *cryIA*(*b*), *cryIA*(*c*) | *cryIA*(*a*), *cryIA*(*c*) | *cryIA*(*b*), *cryIA*(*c*), *cryID* | *cryIC*, *cryID* | *cryIV* |
| Taipei | 3 | 1 | 0 | 0 | 0 |
| Taipei, Yan Ming | 0 | 26 | 0 | 40 | 4 |
| Taichung | 1 | 4 | 6 | 0 | 0 |
| Shieh Shan | 41 | 10 | 0 | 0 | 0 |
| Yu Shan | 82 | 7 | 0 | 0 | 0 |

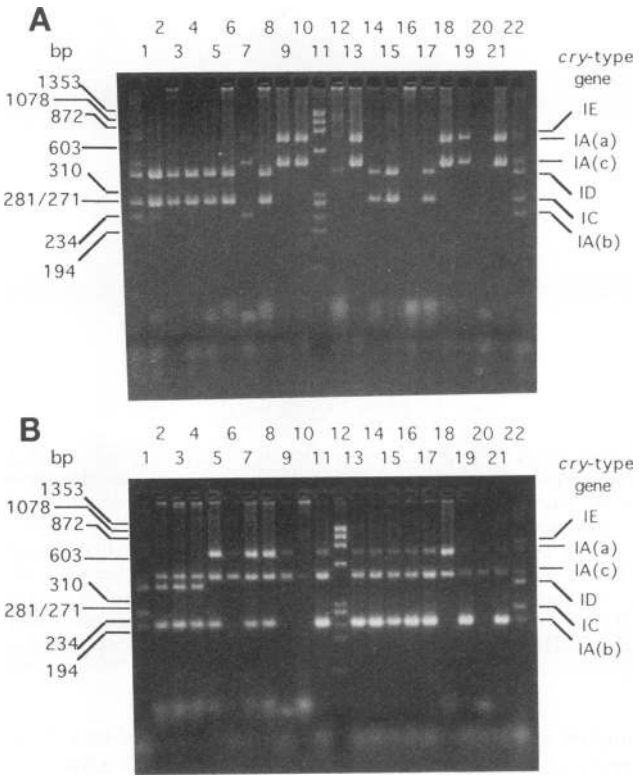


FIG. 4. PCR amplification profiles of *cryI*-type genes of *B. thuringiensis* isolates from Taiwan. Specific PCR-amplified *cryI*-type gene fragments obtained by using the mixed *cryI*-type oligonucleotide primers were run on a 2% agarose gel. Lanes 1 and 22, amplifications of control templates. (A) Lane 11, molecular size markers. Lanes 2 to 10, specific PCR-amplified *cry*-type gene fragments from *B. thuringiensis* isolates TPYM-1, -3, -10, -11, -23, -26, -27, -60, and -61, respectively. Lanes 12 to 21, samples from isolates TPYM-66, -68, -75, -77, -78, -79, -82, -83, -91, and -96, respectively. (B) Lane 12, molecular size markers. Lanes 2 to 6, samples from isolates TC-24, -23, -22, -16, and -6, respectively. Lanes 7 to 10, samples from isolates SS-52, -23, -8, and -143, respectively. Lane 11, sample from isolate YS-132. Lanes 13 to 21, samples from isolates YS-128, -115, -110, -107, -97, -82, -68, -53, and -29, respectively. Positions of molecular size markers and specific amplified *cry*-type gene fragments on the gel are indicated.

*cry* gene profile. *B. thuringiensis* isolates containing *cryIA(a)*-*cryIA(b)*-, and *cryIA(c)*-type genes were found mostly in mountainous areas (Yu Shan and Shieh Shen, 3,000 m above sea level), isolates containing *cryIC*- and *cryID*-type genes were found only on Yang Ming Mountain in Taipei (several hundred meters above sea level), and isolates containing *cryIA(b)*-, *cryIA(c)*-, and *cryID*-type genes were found only in the Taichung area. *B. thuringiensis* isolates containing *cryIA(a)*- and *cryIA(c)*-type genes were common to all isolation areas. We detected no *cryIB*-, *cryIE*-, or *cryIF*-type genes in any isolates.

We used mixed *cryIC*-type oligonucleotide primers (14) (Fig. 5) for PCR to detect the presence of novel *cryIC*-type genes in our isolates. The *cryIC*-type footprints of isolates TPYM-1, -3, -10, -11, -23, -44, -75, and -77 (Fig. 6, lanes 2, 3, 4, 5, 6, 12, 16, and 17, respectively) were distinct from the footprints of the *cryIC(a)* (*B. thuringiensis* subsp. *aizawia* HD229; Fig. 6, lane 22) and *cryIC(b)* (*B. thuringiensis* subsp. *galleriae* HD29; Fig. 6, lane 21) genes, indicating that these *B. thuringiensis* isolates may contain novel *cryIC*-type genes. The *cryIC*-type footprint of *B.*

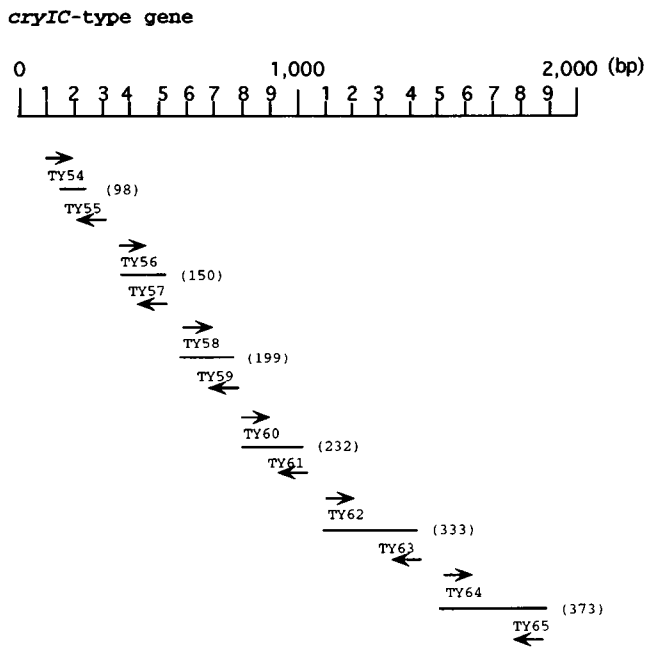


FIG. 5. Strategy for detection of novel *cryIC*-type genes. The relative positions of six pairs of oligoprimers for footprinting of *cryIC*-type genes are shown. Arrows pointing to the right represent forward oligonucleotide primers, whereas arrows pointing to the left represent reverse oligoprimers. Numbers in parentheses are the sizes of specific PCR-amplified fragments.

*thuringiensis* isolates YMB-40 (Fig. 6, lane 11) and TC-22, -23, and -24 (data not shown) resembled that of the *cryIC(a)* gene (Fig. 6, lane 22). In contrast, we detected no *cryIC*-type gene in these isolates when the mixed *cryI*-type oligonucleotide primers were used for PCR to detect the presence of *cryI*-type genes (Fig. 4B, lanes 2, 3, and 4). The detection of *cryIC*-type genes in *B. thuringiensis* isolates by using either the mixed *cryI*-type or mixed *cryIC*-type oligonucleotide primers for PCR is summarized in Tables 3 and 4.

**Insecticidal activity of the *B. thuringiensis* isolates.** Two

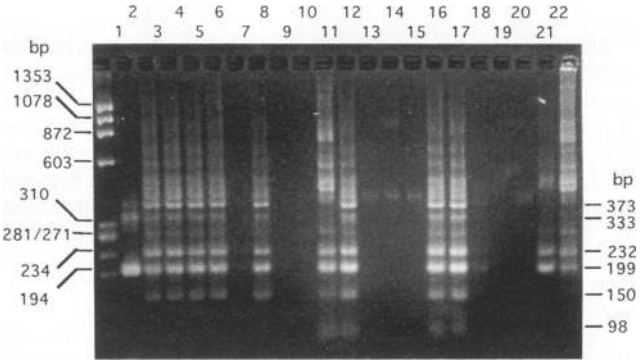


FIG. 6. Footprints of *cryIC*-type genes from *B. thuringiensis* isolates. The footprints were analyzed on a 2% agarose gel. Lane 1, molecular size markers. Lanes 21 and 22, footprints of *cryIC(b)* and *cryIC(a)* genes, respectively. Lanes 2 to 20, footprints of *cryIC* genes from *B. thuringiensis* isolates TPYM-1, -3, -10, -11, -23, -26, -27, -33, -35, -40, -44, -60, -66, -68, -75, -77, -78, -82, and -96, respectively. Positions of molecular size markers and major amplication fragments of *cryIC* footprints are indicated.

TABLE 3. *B. thuringiensis* isolates containing *cryIC*-type genes

| Isolate(s) | Gene(s) obtained with the following PCR amplification oligoprimers: | |
|---|---|---|
| | *cryI* mixed | *cryIC* mixed |
| TPYM-1, -3, -10, -11 | *cryIC*, *cryID* | *cryIC*(*x*) |
| TPYM-23, -27 | *cryIC*, *cryID* | *cryIC*(*y*) |
| TPYM-40 | NT[a] | *cryIC*(*a*) |
| TPYM-44 | NT | *cryIC*(*y*) |
| TPYM-75, -77, -78 | *cryIC*, *cryID* | *cryIC*(*y*) |
| YS-68, -97 | *cryIA*(*a*), *cryIA*(*b*), *cryIA*(*c*) | *cryIC*(?) |
| TC-22, -23, -24 | *cryIA*(*b*), *cryIA*(*c*), *cryID* | *cryIC*(*a*) |

[a] NT, not tested.

hundred *B. thuringiensis* isolates were selected for a primary toxicity test against three locally collected insect species, *P. xylostella*, *H. armigera*, and *S. litura*. Of these isolates, only 23 that showed sufficient toxicity in the tests (Table 5) were selected for further dose-response toxicity tests. In the dose-response toxicity test, only three groups of *B. thuringiensis* isolates exhibited a strong toxicity against *P. xylostella* (Table 6). The insecticidal activities of the isolates against *S. litura* and *H. armigera* were very low (the 50% lethal concentration was above 9,000 ppm).

## DISCUSSION

Using oligoprimers, we identified five profiles of *cry*-type genes, including *cryIA*(*a*)-, *cryIA*(*b*)-, *cryIA*(*c*)-, *cryIC*-, *cryID*-, and *cryIV*-type genes, from 225 *B. thuringiensis* isolates from Taiwan. We observed that the distribution of the *cry*-type genes of our isolates depended on geography. Similar observations were reported (17, 19) for the distributions of *B. thuringiensis* isolates obtained by using serotyping. A profile of *cryIA*(*a*)-, *cryIA*(*b*)-, and *cryIA*(*c*)-type genes was found mostly in *B. thuringiensis* isolated from mountainous areas (at least 3,000 m above sea level), and a profile of *cryIC*- and *cryID*-type genes was found only in the isolates from the Yang Ming Mountain area in Taipei (several hundred meters above sea level). In the Yu Shan and Shieh Shan mountain ranges, the weather is cold and dry, whereas the weather in Taipei is hot and humid. Whether these factors determine the geographical distributions of *cry*-type genes remains to be determined. In contrast, a profile of *cryIA*(*a*)- and *cryIA*(*c*)-type genes was found in all isolation areas. Interestingly, 501 of 536 *B. thuringiensis* isolates (93.5%) were from mountainous areas, in which insects are rare compared with the case in low areas. Although the data were clear, more samples should be collected and examined before a final conclusion on the geographical distribution of *cry* genes is made. PCR provides a facile method to identify the specific *cry*-type genes from a large number of isolates.

Three major *cry*-type gene profiles [*cryIA*(*a*)-, *cryIA*(*b*)-, and *cryIA*(*c*)-type genes; *cryIA*(*a*)- and *cryIA*(*c*)-type genes; and *cryIC*- and *cryID*-type genes] were found in our *B. thuringiensis* isolates. In the assay for high-level primary insecticidal activity in vivo undertaken with various *B. thuringiensis* isolates, we found that isolates containing either the *cryIA*(*a*)-, *cryI*(*b*)-, and *cryI*(*c*)-type gene profile or the *cryIA*(*a*)- and *cryIA*(*c*)-type gene profile exhibited similar insecticidal activities against three tested insects. *B. thuringiensis* isolates containing the *cryIA*(*a*)-, *cryIA*(*b*)-, and *cryIA*(*c*)-type gene profile exerted much greater insecticidal activity against *P. xylostella* in the dose-response in vivo activity test. This discovery indicates that multiple *cry*-type genes may be used as markers for greater insecticidal activity of a *B. thuringiensis* strain.

*B. thuringiensis* isolates containing the *cryIC*- and *cryID*-type gene profile conferred insufficient efficacy against the tested insects in the primary tests. The *cryIC*-type genes confer insecticidal activity against *Spodoptera exigua* (22) and *Spodoptera littoralis* (11), and the *cryID*-type gene is active against *Manduca sexta* (11). We do not know why the *B. thuringiensis* isolate TPYM-23, which contains *cryIC*- and *cryID*-type genes, was inactive against *S. litura* in the primary screening test. Figure 3 shows that the *B. thuringiensis* subsp. *aizawai* strain

TABLE 4. Major specific PCR-amplified fragments observed from the footprints of the *cryIC*-type genes from *B. thuringiensis* isolates

| Gene type | Appearance of the following major PCR fragment (bp) from the *cryIC* footprints[a] | | | | | |
|---|---|---|---|---|---|---|
| | 98 | 150 | 199 | 232 | 333 | 373 |
| *cryIC*(*a*) | + | + | + | + | − | + |
| *cryIC*(*b*) | − | − | + | + | − | − |
| *cryIC*(*x*) | − | − | ++ | − | + | + |
| *cryIC*(*y*) | + | + | + | + | + | + |

[a] ++, with stronger amplification fragment; +, with amplification fragment; −, no amplification fragment.

TABLE 5. High-level primary insecticidal activities of *B. thuringiensis* isolates[a]

| Isolate | Gene types | % Mortality[b] | | |
|---|---|---|---|---|
| | | *P. xylostella* | *H. armigera* | *S. litura* |
| TPYM-23 | *cryIC*, *cryID* | 50 | 20 | 10 |
| TPYM-82 | *cryIA*(*a*), *cryIA*(*c*) | 100 | 90 | 80 |
| TC-16 | *cryIA*(*a*), *cryIA*(*b*), *cryIA*(*c*), *cryIC*[c] | | 73 | 100 |
| TC-19 | *cryIA*(*a*), *cryIA*(*c*) | 100 | 55 | 20 |
| YS-53 | *cryIA*(*a*), *cryIA*(*c*) | 100 | 80 | 100 |
| YS-88 | *cryIA*(*a*), *cryIA*(*b*), *cryIA*(*c*) | 100 | 95 | 100 |
| SS-8 | *cryIA*(*a*), *cryIA*(*c*) | 100 | 95 | 35 |
| SS-23 | *cryIA*(*a*), *cryIA*(*b*), *cryIA*(*c*) | 100 | 75 | 95 |
| SS-52 | *cryIA*(*a*), *cryIA*(*b*), *cryIA*(*c*) | 100 | 95 | |

[a] 10,000 ppm was used for the primary screening test. *B. thuringiensis* subsp. *kurstaki* HD1 was used to define "high level."
[b] Average from three experiments.
[c] The *cryIC*-type gene was detected only by mixed *cryIC*-type oligoprimers.

TABLE 6. Dose-response insecticidal activities of *B. thuringiensis* isolates against *P. xylostella*

| Isolate | Gene types | $LC_{50}$ (ppm)[a] |
|---|---|---|
| TC-19 | *cryIA*(*a*), *cryIA*(*c*) | >250.0 |
| YS-88 | *cryIA*(*a*), *cryIA*(*b*), *cryIA*(*c*) | 128.7 (105.7–156.7) |
| SS-8 | *cryIA*(*a*), *cryIA*(*c*) | >250.0 |
| SS-23 | *cryIA*(*a*), *cryIA*(*b*), *cryIA*(*c*) | 130.1 (106.4–159.5) |
| SS-52 | *cryIA*(*a*), *cryIA*(*b*), *cryIA*(*c*) | 124.6 (101.1–153.6) |
| HD-1[b] | *cryIA*(*a*), *cryIA*(*b*), *cryIA*(*c*) | 131.7 (110.3–157.2) |

[a] The assay was done with spore-crystal complex. Numbers in parentheses indicate 95% confidence limits. $LC_{50}$, 50% lethal concentration.
[b] The *cry*-type gene profile of HD1 is shown in Fig. 3.

contains the *cryIA(a)*-, *cryIA(b)*-, *cryIC*-, and *cryID*-type gene profile. *B. thuringiensis* subsp. *aizawai* strains exhibit high-level insecticidal activity against *Spodoptera* species (11, 22). It seems likely that *cryIA*-type genes coexisting with the *cryIC*-type gene may function together to provide a novel activity against the *Spodoptera* species.

At least two distinct *cryIC*-type PCR footprints were found in our isolates (Tables 3 and 4). The 98-, 150-, and 232-bp PCR fragments were absent in the footprints of the *cryIC(x)*-type gene, but the six major PCR fragments were found in the *cryIC(y)*-type gene. When we used *cryI* mixed oligonucleotide primers to detect the *cry*-type genes of the *B. thuringiensis* isolates, we detected no *cryIC*-type gene in isolates YS-68 and -97 and TC-22, -23, and -24. In contrast, when we used *cryIC* mixed oligonucleotide primers, we observed the footprints of *cryIC(a)*-type genes from those isolates. The *cryIC*-type genes of these strains indicated by the PCR amplifications are somehow different from the *cryIC(a)*-type gene.

Prediction of the insecticidal activity of a newly isolated *B. thuringiensis* strain is important in industry. Because serotypes of *B. thuringiensis* strains are not directly related to the *cry*-type genes in the strains, prediction of the insecticidal activity of *B. thuringiensis* strains by serotyping seems impractical. *B. thuringiensis* subsp. *kurstaki* HD-1 (*cryIA(a)*-, *cryIA(b)*-, *cryIA(c)*-type genes), HD73 (*cryIA(c)*-type gene), and HD263 (*cryIA(b)*- and *cryIA(c)*-type genes) (13) and *B. thuringiensis* isolate TPYM-96 (*cryIA(a)*- and *cryIA(c)*-type genes), which contain different *cry*-type genes, are in the same serotype, 3a:3b. *B. thuringiensis* strains of different serotypes can contain the same *cry*-type genes; for example, *B. thuringiensis* subsp. *alesti* (serotype 3a) and *B. thuringiensis* subsp. *entomocidus* (serotype 6) contain the *cryIA(b)*-type gene, *B. thuringiensis* subsp. *morrisoni* HD-12 (serotype 8a:8b) and *B. thuringiensis* subsp. *toumanoffi* (serotype 11a:11b) contain the *cryID*-type gene, and *B. thuringiensis* subsp. *neoleonensis* (serotype 27) and *B. thuringiensis* subsp. *coreanensis* (serotype 25) contain the *cryIA(a)*-, *cryIA(b)*-, and *cryIA(c)*-type genes (13). *cry* gene typing by PCR amplification therefore seems more useful to predict the insecticidal activity of a *B. thuringiensis* strain, and we propose that typing of *cry*-type genes should be used as one criterion to sort all *B. thuringiensis* strains.

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## ADDENDUM

While we were preparing this paper, a paper reporting the use of PCR to detect *cry*-type genes of *B. thuringiensis* strains was published (3). However, only three *cryIA*-type genes were detected by that method. Our method has the advantage that it can detect all known *cryI*-type genes (*cryIA* type to cryIF type), and we also provide an additional method to detect potential novel *cryIC*-type genes by using six pairs of specific oligoprimers.

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