JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Enterotoxigenicity and Cytotoxicity of *Bacillus thuringiensis* Strains and Development of a Process for Cry1Ac Production

CHI-YEA YANG,† JEN-CHIEH PANG,‡ SUEY-SHENG KAO,§ AND HAU-YANG TSEN*,‡

Department of Food Science, National Chung-Hsing University, Taichung, Taiwan, and Biopesticide Department, Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Taichung, Taiwan

Bacillus thuringiensis is indistinguishable from *Bacillus cereus* except for the production of insecticidal crystal proteins (ICPs). *B. thuringiensis* strains may show enterotoxin profiles and toxin levels similar to those of *B. cereus* strains isolated from food-poisoning cases. It is important for the food industry and farmers to consider that with the application of *B. thuringiensis* strains to crops, their spores may be introduced into the human food chain. In this study, 59 *B. thuringiensis* strains were assayed for their hemolysin BL (HBL) using a BCET-RPLA kit and their cytotoxicity to Chinese hamster ovary (CHO) cells. The enterotoxin titer was as high as that of *B. cereus* diarrheal-type strain ATCC 49064. In an attempt to obtain a food safety strain for bioinsecticide use, in this study, a 3.5-kb *cry1Ac* DNA fragment was amplified with PCR from the total DNA of *B. thuringiensis* subsp. *kurstaki* CCRC 11502 and cloned into the *Bacillus* expression vector pHY300PLK. The α-amylase promoter, *amyE*, was then introduced into the promoter region and, afterward, the recombinant plasmid pHYe1Ac35 was introduced into a non-enterotoxigenic and non-cytotoxic *B. thuringiensis* subsp. *kurstaki* Tt14 strain. The transformant, without any detectable enterotoxigenicity or cytotoxicity, produced Cry1Ac toxin properly, and its insecticidal activity against *Trichoplusia ni* larvae was found to be satisfactory.

KEYWORDS: ***Bacillus thuringiensis*; *cry1Ac*; enterotoxin; food safety; BCET-RPLA kit**

INTRODUCTION

Bacillus thuringiensis is a Gram-positive, endospore-forming bacterium that forms insecticidal crystal proteins (ICPs) during the sporulation phase (*1*) and has been used for bioinsecticide production for many decades. On the other hand, *Bacillus cereus* is one of the common food pathogens that often produce enterotoxins. It is cytotoxic and has been implicated in food-poisoning cases with emetic or diarrheal syndromes (*2*). Of the major enterotoxins produced by *B. cereus*, hemolysin BL (HBL), enterotoxin T (BceT), and enterotoxin FM (Ent FM) have been purified, and their genes have been cloned (*3*−*5*). Of them, HBL enterotoxin is the most common target for the measurement of *B. cereus* enterotoxigenicity. For such an assay, a commercial detection kit, that is, a *B. cereus* enterotoxin reversed passive latex agglutination (BCET-RPLA) test kit, is commercially available. It is now apparent that *B. thuringiensis* and *B. cereus* are often considered to be members of the same species on the basis of the similarity of their phenotypic properties, 16S and 23S rRNA gene sequences, common enterotoxin profiles, and comparable toxicity levels (*6*−*8*). *B. thuringiensis* has also been found to be involved in outbreaks of gastrointestinal disease (*9*).

On the basis of the above-described facts, it is thus important for the bioinsecticide industry to consider that although *B. thuringiensis* insecticide has been used for many years, *B. thuringiensis* may be introduced into the human food chain through the application of this bacteria species to crops. Although the production of enterotoxins by *B. thuringiensis* may be affected by the culture medium, time, and fermentation conditions (*10*), introduction of *B. thuringiensis* spores into the human food chain followed by spore regermination may cause a risk of food-borne poisoning cases (*11*). Accordingly, to reduce such a food-poisoning risk, it is better to isolate a non-enterotoxigenic *B. thuringiensis* strain for bioinsecticide production or to consider undertaking the simple expedient of deleting those enterotoxin genes from the commercial strains for the production of insecticidal crystal toxins. Because it is difficult (<0.05%) to find a non-enterotoxigenic bioinsecticide producing *B. thuringiensis* strain from the environment (*10*), and also difficult to obtain an ideal crystal toxin producing vector from other laboratories, in this study, we tried to construct a Cry1Ac producing vector and introduce it into a non-enterotoxigenic and non-cytotoxic *cry*⁻ *B. thuringiensis* strain. Although such

* Address correspondence to this author at the Department of Food Science, National Chung-Hsing University, 250 Kuo-Kuang Rd., Taichung, Taiwan (telephone +886-4-22870162; fax +886-4-22870162; e-mail hytsen@dragon.nchu.edu.tw).

† Present address: Department of Cosmetic Science, Van-Nung Institute of Technology, Chungli City, Taoyuan, Taiwan (e-mail paulyea123@cc.vit.edu.tw).

‡ National Chung-Hsing University.

§ Taiwan Agricultural Chemicals and Toxic Substances Research Institute.

10.1021/jf025863l CCC: $25.00 © 2003 American Chemical Society
Published on Web 12/07/2002

a process is rather obvious, an amylase promoter fragment, *amyE*, was fused into the promoter region to induce the expression of *cry1Ac* at the early log phase instead of the *B. thuringiensis cry* replicon, which was promoted only at sporulation stage.

MATERIALS AND METHODS

Bacterial Strains. The *Bacillus* strains used were obtained from the American Type Culture Collection (ATCC) in Rockville, MD; the Culture Collection and Research Center (CCRC) in Hsin-Chu, Taiwan; and the Taiwan Agriculture Chemical and Toxic Substances Research Institute (TACTRI) in Taichung, Taiwan. The *B. thuringiensis* strains were grown at 30 °C in Luria−Bertani (LB) or casitone−yeast−sucrose (CYS) (*12*) medium while shaking for 12 h or until the liberation of spores and crystal toxins. Strains of *B. thuringiensis* subsp. *kurstaki* CCRC 11502 and Tt14 from TACTRI were used for total DNA preparation and as final host for the expression of the *cry1Ac* gene, respectively.

Five commercial products of *B. thuringiensis*-based insecticides coded A, B, C, D, and E were used in this study. *B. thuringiensis* was isolated by transferring a loop of each of the products to a nutrient agar plate (Difco), followed by incubation at 30 °C for sporulation. *Escherichia coli* DH5α was used as the host for transformation of recombinant plasmids. The above-described *Bacillus* or *E. coli* strains were generally maintained at −70 °C as suspensions of cells or spores in 10% (v/v) glycerol. The miniaturized biochemical API 50 CHB (bioMerieux) kit was used to confirm the *Bacillus* species. This method was unable to differentiate *B. thuringiensis* from *B. cereus*. In such a case, additional tests, such as 1000× microscope observation for the crystal toxins, were performed.

Enterotoxin Detection by BCET-RPLA. Enterotoxigenic activity was analyzed with the *B. cereus* enterotoxin (diarrheal type) reversed passive latex agglutination kit (BCET-RPLA) (Denka Seiken, Tokyo, Japan). This kit is specific for the L2 component of *B. cereus* hemolysin BL enterotoxin (*13*). Assay was performed according to the manufacturer's instruction. One loop of 24 h LB culture broth was inoculated into 5 mL of BHIG medium (0.04% brain heart infusion and 0.1% glucose), and the mixture was incubated at 30 °C for 12 h. Then the culture was centrifuged for 5 min at 5000*g* (Himac SCR20B, Hitachi, Tokyo, Japan), and the cell-free supernatants were subjected to enterotoxin detection with the BCET-RPLA kit.

Cytotoxicity Assay. Cell culture assay used in this study was modified from the methods described by Buchanan and Schultz (*13*). Chinese hamster ovary (CHO) cells were maintained in McCoy's 5A medium containing 10% fetal bovine serum, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin and incubated at 37 °C in an atmosphere containing 5% CO_2. Cell monolayers were treated with 1× trypsin−EDTA. The cell density was adjusted to 5×10^5−10^6 cells mL^{-1} with McCoy's 5A medium, and 50 μL of the cell suspension was seeded into each of the 96 wells of the microtiter plate. Monolayers were established within 18−20 h. The 12 h cell-free culture supernatant of *B. thuringiensis* strains was prepared by centrifugation of the cell culture at 5000*g* for 15 min followed by passing the supernatant through a 0.45 μm filter (Millipore, Bedford, MA). Fifty microliters of the 2-fold serial dilutes of cell-free supernatants was pipetted into each well. After incubation at 37 °C for 24 h, the plate was emptied and 50 μL of 2% formalin was added to each well and allowed to stand at room temperature for 1 min. After fixation with formalin, each well was stained for 20 min with 50 μL of 0.13% crystal violet in PBS buffer containing 5% ethanol and 2% formalin and, then, rinsed three times with sterile water followed by air-drying. Afterward, 50 μL of 50% ethanol was added and the crystal violet was allowed to solubilize for 1 h. The absorbance was determined at 630 nm by using an ELISA reader (EL340, Bio-Tek, Winooski, VT). Optical density measurements allowed estimation of the remaining monolayer cell number, and the reaction was considered to be positive when >50% of the cells detached. The results were recorded according to the manufacturer's instructions. The 2^0-fold dilution represents the result from negative control, and the 2^8-fold dilution represents a strong reaction, such as that from *B. cereus* ATCC 49064.

Vectors and Cloning of *cry1Ac*. The chromosomal DNA was prepared from 5 mL of overnight culture of *B. thuringiensis* by the use of a Puregene DNA isolation kit (Philip Harris/Flowgen, Shenstone, U.K.). Plasmid pGEM3Zf (+) was used to clone the *cry1Ac* fragment that was obtained by the following process using primer cry1Ac-F (5′-gcggatcctatggataacaatc), a forward primer for the 5′ end, and a reverse primer cry1Ac−R (5′-tagtcgacactattcctccat) for the command 3′ end. A complete protein-coding region of *cry1Ac* of ~3.5-kb was amplified from the total DNA of *B. thuringiensis* subsp. *kurstaki* CCRC 11502 with cry1Ac-F and cry1Ac-R primers, and through the use of these PCR primers, the *Bam*HI and *Sal*I cutting sites were created at the 5′ and 3′ ends of the fragment. The PCR conditions were 1 cycle of 4 min at 94 °C, 35 cycles of 30 s at 94 °C, 40 s at 50 °C, and 70 s at 72 °C, and a final extension of 7 min at 72 °C. Amplification reactions were performed on a Perkin-Elmer 9600 thermocycler (Norwalk, CT). The PCR mixture (25 μL) consisted of 200 μmol L^{-1} each of dATP, dGTP, dTTP, and dCTP, 1× PCR buffer (10 mmol L^{-1} Tris-HCl, pH 8.8, 1.5 mmol L^{-1} $MgCl_2$, 50 mmol L^{-1} KCl, and 0.1% Triton X-100), 25 pmol each of the primers, ~50 ng of chromosomal DNA, and 0.4 unit of Dynazyme (Finnzyme, Riihitontuntie, Finland). The PCR products were analyzed by electrophoresis with 2% agarose gel. On the other hand, an amylase promoter, *amyE*, containing the ribosome-binding site, was amplified from plasmid pSB744 (*14*) with primers amyE-F (5′-atgaattccagggctgcggcatc) and amyE-R (5′-atggatccatgggt-tacctccatc). The restriction enzyme sites of *Eco*RI and *Bam*HI were also introduced through the primers into the 5′ and 3′ ends of the *amyE* PCR product. Such a PCR fragment was cloned into another pGEM3Zf (+) to construct the pGEM-ebE plasmid.

The 3.5-kb *cry1Ac* DNA fragment described earlier was cloned into a pGEM3Zf (+) vector to construct the pGEM1Ac35 plasmid. The nucleotide sequence of this DNA fragment was determined and was found to be identical to the *cry1Ac* sequence published by Gleave et al. (*15*). The *cry1Ac* DNA fragment of pGEM1Ac35 was subcloned into the *Bacillus* expression vector pHY300PLK (Takara, Tokyo, Japan) to construct pHY1Ac35, and then the *amyE* promoter fragment obtained from described earlier was fused into the promoter region of the recombinant plasmid pHY1Ac35 (**Figure 1**). Such a recombinant plasmid was termed pHYe1Ac35 and was electroporated into *B. thuringiensis* subsp. *kurstaki* Tt14 by the use of an electroporater (Equibio, Easyject Prima, Kent, U.K.). Strain *B. thuringiensis* subsp. *kurstaki* Tt14 obtained from TACTRI is a plasmid-negative strain that has been identified as a non-enterotoxigenic and non-cytotoxic strain.

Molecular Techniques. Plasmid DNA was extracted and analyzed according to a procedure modified from the method of Kado and Liu (*16*). Molecular techniques used in this study include restriction enzyme digestion, DNA ligation, sequence analysis, and transformation. The approaches used were those described by Sambrook et al. (*17*).

Isolation of Cry1Ac Protein. Crystal toxins were harvested from the culture medium and purified according to the procedures described by Lopez-Meza and Ibarra (*18*). *B. thuringiensis* strains were grown in 100 mL of CYS medium until the spores and crystal toxins were liberated completely into the medium. The spores and crystal toxins were harvested and washed three times with washing solution (0.5 M NaCl and 2 mM EDTA). Subsequently, the washed pellet was resuspended in 5 mL of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) containing 0.3 M mercaptoethanol and 0.5 M phenylmethanesulfonyl fluoride. The crystal toxins were solubilized by adjusting the pH to >10.5 with 2 N NaOH. The insoluble fraction was removed by centrifugation at 20000*g* for 10 min. Supernatant containing the crystal toxins was adjusted to pH 4.4 with 2 N HCl and kept in an ice bath for 60 min to precipitate the crystal toxins. Afterward, the crystal toxins were collected by centrifugation at 10000*g* for 10 min. After the pellet had been dissolved in 0.1 M sodium phosphate buffer (pH 7.0), the protein concentration was determined with the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Molecular weights of the proteins were determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE).

Bioassays against *Trichoplusia ni* Larvae. Bioassays were performed against the laboratory-reared insect species *T. ni*. The 7 day culture of *B. thuringiensis* was centrifuged at 7000*g* for 10 min, and the pellets containing spores and crystal toxins were washed with sterile

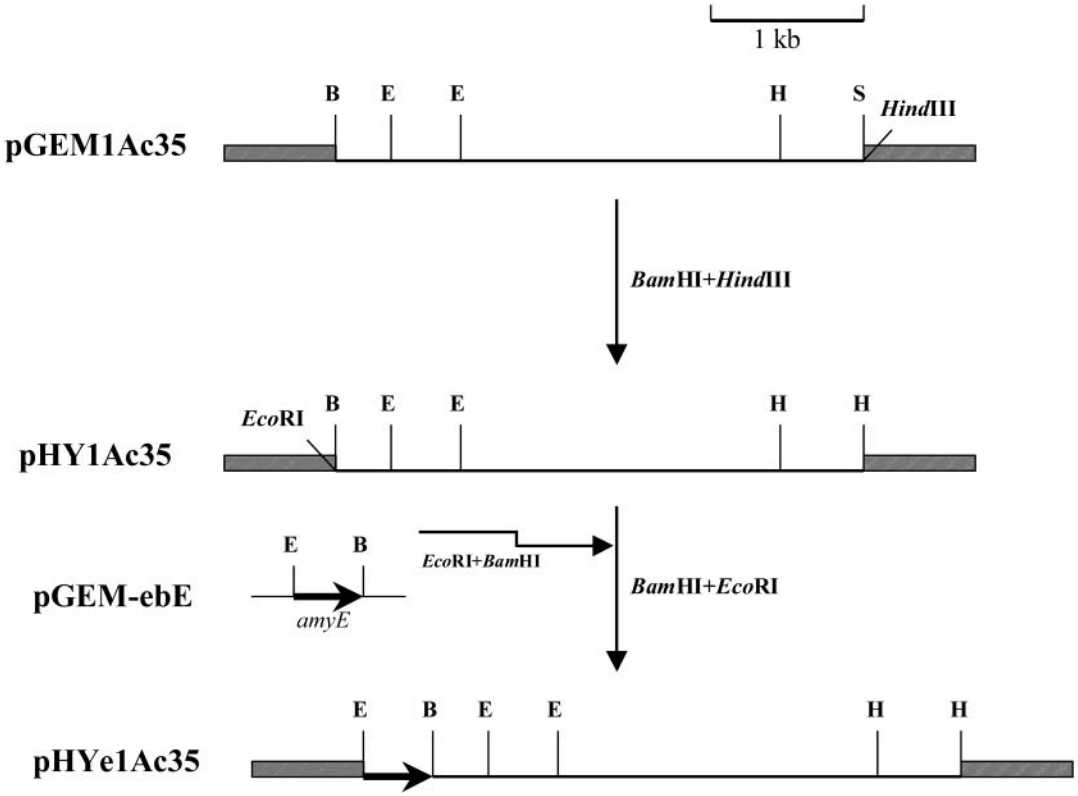

Figure 1. Construction of plasmid pHYe1Ac35. A 3.5-kb fragment (base line) containing the *cry1Ac* coding region was amplified by the PCR method and cloned into the *Bam*HI and *Sal*I sites of pGEM3Zf(+) to construct pGEM1Ac35. Partial digestion of pGEM1Ac35 with *Hin*dIII and *Bam*HI generates a 3.5-kb *Bam*HI–*Hin*dIII DNA fragment, which was then subcloned into pHY300PLK to construct pHY1Ac35. The hatched arrowhead indicates the reading orientation of the *amyE* promoter containing the ribosomal binding sequence, and it was introduced into the promoter region of the pHY1Ac35 to construct pHYe1Ac35. B, E, H, and S are restriction sites of *Bam*HI, *Eco*RI, *Hin*dIII, and *Sal*I, respectively.

Table 1. Titer of *Bacillus* Diarrheal Enterotoxin and Cytotoxin of 12 h Cultures[a]

strain[b]	no. of tested strains	BCET-RPLA[c]		cytotoxicity		crystal[d]
		no. of positive strains	titer	no. of positive strains	titer	
B. thuringiensis						
ATCC strains (10729, 33679, 13367, 14713)	4	4	2^{11-12}	4	2^{7-8}	+
CCRC strains (11501, 11502, 14373–14377, 15070)	8	8	2^{11-12}	8	2^{7-8}	+
isolates from TACTRI (Tt16–Tt62)	47	47	2^{11-12}	47	2^{7-8}	+
isolates from commercial product						
A	1	1	2^{9}	1	2^{8}	+
B	1	1	2^{11}	1	2^{8}	+
C	1	1	2^{9}	1	2^{8}	+
D	1	1	2^{9}	1	2^{6}	+
E	1	1	2^{10}	1	2^{7}	+
B. cereus						
ATCC strains (7004, 11143, 11778, 13061, 14579, 19637, 21182, 21281, 21769, 21771, 49064[e])	11	5	2^{11-12}	5	2^{7-8}	–
CCRC strains (10250, 10282, 11026, 11835, 11833, 11834, 11910, 11913, 14194–14197, 14655, 14689, 14699)	15	11	2^{11-12}	11	2^{7-8}	–
B. subtilis CCRC 10029[f]	1	1	2^{0}	1	2^{0}	–

[a] Experimental conditions were as described under Materials and Methods. The results shown are according to the manufacturer's instructions. 2^{n} means the dilution titer, which still allows the positive reaction. For example, a 2^{0}-fold dilution means a weak reaction, whereas a 2^{12}-fold dilution means a strong reaction. [b] All tested strains were described under Materials and Methods. [c] BCET-RPLA is specific to detect the L2 component of *B. cereus* hemolysin BL. [d] Crystal toxins were observed under the 1000× microscope. [e] *B. cereus* ATCC 49064 was the diarrheal-type strain. [f] *B. subtilis* CCRC 10092 served as the negative control.

distilled water twice. Then the spore−crystal mixtures were resuspended in a $^{1}/_{10}$ volume of sterile distilled water. The spore−crystal mixtures to be assayed were sprayed onto the 1 g of artificial insect solid diet (*19*) at different concentrations and fed to the second-instar larvae at 25 °C. All doses were repeated three times using 30 neonate larvae per assay. Mortality was recorded after incubation for 3 days under insectary conditions, and the 50% lethal concentration (LC_{50}) was determined by Probit analysis (*20*) based on at least three independent bioassays.

RESULTS AND DISCUSSION

Enterotoxigenicity and Cytotoxicity. For the 59 *B. thuringiensis* and 26 *B. cereus* strains shown in **Table 1**, results from the BCET-RPLA assay were agreeable with those from the cytotoxicity assay. All (100%) the 59 *B. thuringiensis* strains were HBL positive and cytotoxic to CHO cells. Meanwhile, only 5 of the 11 ATCC and 11 of the 15 CCRC *B. cereus* strains (62%) were HBL positive, and all of these HBL-positive strains

Table 2. Enterotoxicity, Cytotoxicity, and Insecticidal Activity Assay of *B. thuringiensis*, Its Transformant, and *B. cereus*

assay	Tt14[a]	pHYeIAc35/ Tt14	Bt4[b] (CCRC 11502)	Bc1[c] (ATCC 49064)	Bs[d]
enterotoxicity (BCET-RPLA)	2^1	2^0	2^{12}	2^{12}	2^0
cytotoxicity	2^1	2^1	2^7	2^8	2^1
LC_{50}[e] (ppm)	>15000	185 ± 13	165 ± 11	>15000	>15000

[a] *B. thuringiensis* subsp. *kurstaki* Tt14 is a *cry*$^-$ strain. [b] *B. thuringiensis* subsp. *kurstaki* CCRC 11502 is a crystal toxin production strain. [c] *B. cereus* diarrheal-type strain ATCC 49064 served as the positive control for the enterotoxicity test. [d] *B. subtilis* CCRC 10029 served as the negative control. [e] LC_{50}, 50% lethal concentration against *Trichoplusia ni*.

were cytotoxic to CHO cells. Also, the enterotoxin titers of *B. thuringiensis* strains were as high as that of *B. cereus* diarrheal-type strain (ATCC 49064) (**Table 1**). In addition, all of the *B. thuringiensis* strains isolated from the imported commercial available *B. thuringiensis*-based insecticides purchased in Taiwan, that is, products A−E, were shown to produce HBL and were cytotoxic to CHO cells (**Table 1**). These results indicated that strains from these products can produce diarrheal enterotoxin. Results in **Table 2** indicate that as assayed with the BCET-RPLA kit, the undiluted supernatants from the cultures of *B. thuringiensis* subsp. *kurstaki* Tt14, a *cry*$^-$ strain, and its transformant harboring pHYe1Ac35 generated negative results. Such a result was similar to that of strain *B. subtilis* CCRC 10029, which was used as negative control. On the other hand, strain *B. thuringiensis* subsp. *kurstaki* CCRC 11502 was highly enterotoxigenic and cytotoxic. For its 12 h culture broth, even after 2^{12}-fold dilution, the enterotoxins were detectable. Such a titer was as high as that of the *B. cereus* diarrheal-type strain ATCC 49064.

B. thuringiensis produces enterotoxins similar to those of *B. cereus* and, thus, was also a potential hazard for food products (*21*, *22*). Gaviria et al. (*21*) pointed out that there is little difference between the distribution of enterotoxin genes among strains of *B. thuringiensis* and *B. cereus* and, furthermore, these genes are more common for the former. We also found that most of the *B. thuringiensis* strains showed high levels of enterotoxigenicity and cytotoxicity, equivalent to that associated with *B. cereus* strains isolated from diarrheal food-poisoning cases. Introduction of the *B. thuringiensis* spores into the human food chain followed by regermination may lead to the food poisoning. Accordingly, although boiling for 12 min could reduce the diarrheal enterotoxigenicity (*10*), the discovery of a *B. thuringiensis* strain without enterotoxigenicity and cytotoxicity for the production of Cry toxin is important from the viewpoint of food or crop safety.

Construction of the Cry1Ac Producing Vector. Steps for the preparation of *cry1Ac* coding region fused with *amyE* promoter are shown in **Figure 1**. The nucleotide sequences of the PCR primers used for *cry1Ac* amplification were designed from the sequences published by Gleave et al. (*15*). The expressed Cry1Ac toxin of pGEM1Ac35 in *E. coli* DH5α was analyzed by SDS-PAGE, and the cell-free crude extract showed that the amount of Cry1Ac toxin produced with *lac* promoter in *E. coli* was significantly lower (50%) than that from the strain of *B. thuringiensis* subsp. *kurstaki* CCRC 11502 (data not shown). To improve the expression of the *cry1Ac* gene in *B. thuringiensis*, the Cry1Ac protein-coding region was subcloned into pHY300PLK, and the resulting plasmid was called pHY1Ac35. After the introduction of the *amyE* promoter, the recombinant plasmid was termed pHYeIAc35, and it was assured by restriction enzyme digestion and DNA sequencing.

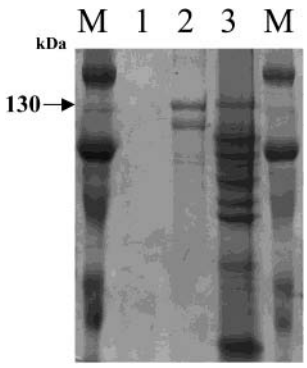

Figure 2. Coomassive blue-stained SDS-PAGE for Cry toxins prepared from *B. thuringiensis* Tt14 (lane 1), *B. thuringiensis* Tt14 harboring the recombinant plasmid pHYe1Ac35 (lane 2), and *B. thuringiensis* subsp. *kurstaki* CCRC 11502 (lane 3). Twenty microliters of each sample was loaded onto the 8% SDS-PAGE gel, electrophoresis was performed at 200 V for 90 min. M, molecular weight markers (Rainbow, RPN756). Band position for the 130 kDa Cry1Ac protoxin is indicated.

Expression of different kinds of crystal toxins in *B. thuringiensis* is directly related to their toxicity to the insects. Many studies have been proposed to achieve a high yield of *B. thuringiensis* toxins, such as putting the *cry* gene under the control of an effective promoter, increasing the gene copy number, or promoting the gene coexpression (*23*−*25*). On the other hand, although a helper protein P20 (a 20-kDa protein), an accessory protein found in *B. thuringiensis* subsp. *israelensis*, has been discovered during the study of Cyt1A (cytolytic crystal protein) expression and found to be essential for the efficient production of Cyt1A (*25*), Chang et al. (*26*) hypothesized that the high-level expression of *cyt1A* and *cryIVD* in *B. thuringiensis* does not require P20 but requires other coexpressed proteins. Lee and Gill (*23*) also reported that P20 failed to have a promoting effect on *cry20Aa*. Recently, Shao et al. (*24*) found that P20 could enhance the production of full-length Cry1Ac protoxin under the control of a *B. thuringiensis* replicon that was regulated under the sporulation steps. Due to the difficulty of obtaining such a recombinant plasmid and the uncertainty pf whether P20 could properly serve as a helper protein for the *amyE* promoter, used in this study, we constructed the recombinant plasmid pHYe1Ac35 not including the gene of helper protein P20.

Expression of the *cry1Ac* Gene in *B. thuringiensis*. The recombinant plasmid pHYe1Ac35, carrying the α-amylase promoter, *amyE*, and the Cry1Ac protein-coding region, was electrotransformed into the *B. thuringiensis* subsp. *kurstaki* Tt14 that was essentially a non-enterotoxigenic and *cry*$^-$ strain. After 2 days of culture at 30 °C, *B. thuringiensis* subsp. *kurstaki* Tt14 transformant harboring the pHYe1Ac35 could be screened out by its tetracycline-resistant activity. The transformants grew slowly in LB broth when the tetracycline concentration was >5 μg/mL. However, under such a condition, the recombinant plasmid pHYe1Ac35 was stably maintained.

Crystal proteins were purified from cells harboring the recombinant plasmid by acid precipitation (*18*). *B. thuringiensis* subsp. *kurstaki* CCRC 11502 could produce a 130 kDa protein, whereas the *B. thuringiensis* subsp. *kurstaki* Tt14 transformant harboring the pHYe1Ac could, too (**Figure 2**). Because formation of the crystal toxin, which occurs during the sporulation of *B. thuringiensis*, correlates with high protease activity, and the digestion of crystal toxins by protease is different between

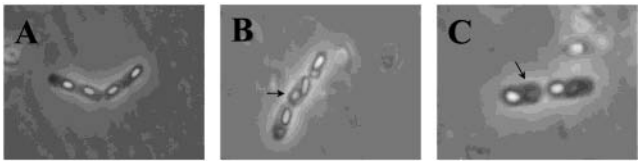

Figure 3. Expression of *cry1Ac* in *B. thuringiensis*: (A) *B. thuringiensis* subsp. *kurstaki* Tt14 (host cell); (B) *B. thuringiensis* subsp. *kurstaki* CCRC 11502; (C) *B. thuringiensis* Tt14 transformant harboring the constructed pHYe1Ac. Insecticidal crystal proteins (ICP) could be observed under the 1000× microscope, and the crystals are indicated by arrows.

isolates (*27*), some of the 130 kDa Cry1Ac produced from *B. thuringiensis* subsp. *kurstaki* Tt14 transformant was proteolyzed to shorter peptides (**Figure 2**), whereas the Cry toxins of *B. thuringiensis* subsp. *kurstaki* CCRC 11502, which harbored three different *cry* genes, were proteolyzed to several smaller polypeptides (**Figure 2**). These polypeptides (protoxins) could be later further converted to active toxins by the larval gut proteases (27).

Expression of the *cry* gene in *B. thuringiensis* was a sporulation-regulated event. In the recombinant *B. thuringiensis* subsp. *kurstaki* Tt14 transformant, expression of the *cry1Ac* gene was regulated under the promoter, *amyE*, which required a specific sigma factor, ζ^{43}, whereas for the other *cry* genes, ζ^{28} and ζ^{35} were required (*28*). Also, for the *amyE* promoter, the transcription activity increased substantially when cell growth approached to the late log phase. This means that expression of the *cry1Ac* gene, under the regulation of *amyE*, starts from the early growth phase to the late log phase (*14*). Thus, the quantity of Cry toxin (67 ± 4 μg mL^{-1}) produced from the transformant of *B. thuringiensis* subsp. *kurstaki* Tt14, which harbored only a *cry1Ac* gene, was somewhat higher than the quantity (58 ± 5 μg mL^{-1}) of the total Cry toxins produced from the *B. thuringiensis* subsp. *kurstaki* CCRC 11502, which synthesized three different Cry toxins during the sporulation stage. This result demonstrated that the *cry1Ac* gene could be expressed efficiently with the *amyE* promoter in the transformant of *B. thuringiensis* subsp. *kurstaki* Tt14.

Insecticidal Activity. In many instances, the spore crystal preparation is much more toxic than purified proteins (*1*); thus, the insecticidal activities of *B. thuringiensis* subsp. *kurstaki* CCRC 11502 and Tt14 transformant harboring pHYe1Ac35 were determined by measuring their LC_{50} values against *T. ni* larvae using their washed spore crystal preparations. **Figure 3** shows that both *B. thuringiensis* subsp. *kurstaki* CCRC 11502 and *B. thuringiensis* subsp. *kurstaki* Tt14 transformant properly produced crystal toxins after 48 h of culture and that all the spores and crystal toxins were liberated after 96 h of culture in the CYS medium. Although *B. thuringiensis* subsp. *kurstaki* CCRC 11502 ($LC_{50} = 165 \pm 11$ ppm) produced a lower quantity of acid-precipitated protein, it showed a little bit higher level of insecticidal activity against *T. ni* than did the strain of *B. thuringiensis* Tt14 transformant harboring pHYe1Ac ($LC_{50} = 185 \pm 13$ ppm) (**Table 2**). This may be due to the fact that the insecticidal activity of *B. thuringiensis* is dependent on the quantity and the types of δ-endotoxin (*1*); *B. thuringiensis* subsp. *kurstaki* CCRC 11502 harbored three different *cry* genes, one of which was the *cry1Ac* gene. Also, the synergistic effects of different crystal toxins are important. Gleave et al. (*15*) reported that the interactions of toxins found in *B. thuringiensis* subsp. *israelensis* revealed evident synergism when the toxins act in different combination against several kinds of larvae. On the other hand, Mohan and Gujar (*29*) reported that the insecticidal activity of Biobit (*B. thuringiensis* subsp. *kurstaki* HD1) (Rallis India Ltd., Mumbai, India), a commercial bioinsecticide containing five different *cry* genes, was dependent on the populations of larvae obtained from different countries or areas. Its LC_{50} may range from 1 to 10 ppm. In this study, the LC_{50} values of *B. thuringiensis* subsp. *kurstaki* CCRC 11502 and Tt14 transformant were 165 ± 11 and 185 ± 13 ppm, respectively. These are much higher than that of the Biobit but agree with the results of Chak et al. (*14*), against the same larva, *T. ni*. This may be due to the fact that *B. thuringiensis* subsp. *kurstaki* HD73, used in the study of Chak et al., also harbors only one *cyr1Ac* gene. Such results demonstrate that the Cry1Ac toxin produced from *B. thuringiensis* subsp. *kurstaki* Tt14 transformant through the expression system described in this paper exhibits proper insecticidal activity against *T. ni*.

Because foods may be contaminated by the spores of *B. thuringiensis* or *B. cereus* (*30*), it is important that cooked foods for use in salads or other dishes be stored in such a way to prevent spore germination or bacterial growth. From the viewpoint of food safety, it is better to use a non-enterotoxigenic and non-cytotoxic *B. thuringiensis* strain for the bioinsecticide production. Finally, we have to point out that, although introduction of a crystal toxin producing vector in a non-enterotoxigenic *B. thuringiensis* strain is a rather obvious process and the *B. thuringiensis* transformant reported here may not be the most ideal one for bioinsecticide production, this paper showed an example of how to obtain a food safety *B. thuringiensis* strain that may be used for bioinsecticide production. It should be remembered that in addition to the approaches described here for plasmid construction and gene cloning, other *cry* genes, for example, *cry1Ab*, other crystal toxin producing vectors, or other hosts may be used for the production of non-enterotoxigenic and non-cytotoxic bioinsecticides.

ACKNOWLEDGMENT

We are grateful to TACTRI, Taichung, Taiwan, for providing *B. thuringiensis* subsp. *kurstaki* Tt14 and, also, technical assistance for the insecticide activity assay.

LITERATURE CITED

(1) Schnepf, E.; Crickmore, N.; Van, R. J.; Lereclus, D.; Baum, J.; Feitelson, J.; Zeigler, D. R.; Dean, D. H. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 775−806.

(2) Notermans, S.; Batt, C. A. A risk assessment approach for foodborne *Bacillus cereus* and its toxins. *J. Appl. Microbiol. Symp. Suppl.* **1998**, *84*, 51s-61s.

(3) Beecher, D. J.; Wong, A. C. Tripartite hemolysin BL from *Bacillus cereus*. Hemolytic analysis of component interaction and model for its characteristic paradoxical zone phenomenon. *J. Biol. Chem.* **1997**, *272*, 233−239.

(4) Beecher, D. J.; Schoeni, J. L.; Wong, A. C. Enterotoxin activity of hemolysin BL from *Bacillus cereus*. *Infect. Immun.* **1995**, *63*, 4423−4428.

(5) Lund, T.; Granum, P. E. Characterization of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a food-borne outbreak. *FEMS Microbiol. Lett.* **1996**, *141*, 151−156.

(6) Ash, C.; Collins, M. D. Comparative analysis of 23S ribosomal RNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-direct sequencing. *FEMS Microbiol. Lett.* **1992**, *94*, 75−80.

(7) Barjac, H.; Frachon, E. Classification of *Bacillus thuringiensis* strains. *Entomophaga* **1990**, *35*, 233−240.

(8) Carlson, C. R.; Johansen, T.; Kolsto, A. B. The chromosome map of *Bacillus thuringiensis* subsp. *canadensis* HD244 is highly similar to that of *Bacillus cereus* type strain ATCC 14579. *FEMS Microbiol. Lett.* **1996**, *141*, 163−167.

(9) Perani, M.; Bishop, A. H.; Vaid, A. Prevalence of β-exotoxin, diarrhoeal toxin and specific δ-endotoxin in natural isolates of *Bacillus thuringiensis*. *FEMS Microbiol. Lett*. **1998**, *160*, 55−60.
(10) Damgaard, P. H. Diarrhoeal enterotoxin production by strains of *Bacillus thuringiensis* isolated from commercial *Bacillus thuringiensis*-based insecticides. *FEMS Immunol. Med. Microbiol.* **1995**, *12*, 245−250.
(11) Hansen, B. M.; Hendriksen, N. B. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Appl. Environ. Microbiol.* **2001**, *67*, 185−189.
(12) Yamamoto, T. Identification of entomocidal toxins of *Bacillus thuringiensis* by high-performance liquid chromatography. In *Analytical Chemistry of Bacillus thuringiensis*; Hickle, L. A., Fitch, W. L., Eds.; American Chemical Society: Washington, DC, 1990; pp 46−60.
(13) Buchanan, R. L.; Schultz, F. J. Evaluation of the Oxoid BCET-RPLA kit for the detection of *Bacillus cereus* diarrhoeal enterotoxin and compared to cell culture cytotoxicity. *J. Food Prot.* **1992**, *55*, 440−443.
(14) Chak, K. F.; Tseng, M. Y.; Yamamoto, T. Expression of the crystal protein gene under the control of the α-amylase promoter in *Bacillus thuringiensis* strains. *Appl. Environ. Microbiol.* **1994**, *60*, 2304−2310.
(15) Gleave, A. P.; Hedges, R. J.; Broadwell, A. H.; Wigley, P. J. Cloning and nucleotide sequence of an insecticidal crystal protein gene from *Bacillus thuringiensis* DSIR732 active against 3 species of leafroller (Lepidoptera, Tortricidae). *N.Z. J. Crop Hortic. Sci.* **1992**, *20*, 27−36.
(16) Kado, C. I.; Liu, S. T. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* **1981**, *145*, 1365−1373.
(17) Sambrook, J.; Fritch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.
(18) Lopez-Meza, J. E.; Ibarra, J. E. Characterization of a novel strain of *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **1996**, *62*, 1306−1310.
(19) Graham, D. Y.; Conlong, D. E. Improved laboratory rearing of *Eldana saccharina* (Lepidoptera: Pyralidae) and its indigenous parasitoid *Goniozus natalensis* (Hyenoptera: Bethylidae) *Proc. S. Afr. Sugar Technol. Assoc. Annu. Congr*. **1988**, *62*, 116−119.
(20) Finney, D. J. *Probit Analysis*, 3rd ed.; Cambridge University Press: Cambridge, U.K., 1977.
(21) Gaviria, R. A. M.; Granum, P. E.; Priest, F. G. Common occurrence of enterotoxin genes and enterotoxicity in *Bacillus thuringiensis*. *FEMS Microbiol. Lett.* **2000**, *190*, 151−155.
(22) Hsieh, Y. M.; Sheu, S. J.; Chen, Y. L.; Tsen, H. Y. Enterotoxigenic profiles and polymerase chain reaction detection of *Bacillus cereus* group cells and *B. cereus* strains from foods and foodborne outbreaks. *J. Appl. Microbiol.* **1999**, *87*, 481−490.
(23) Lee, H. K.; Gill, S. S. Molecular cloning and characterization of a novel mosquitocidal protein gene from *Bacillus thuringiensis* subsp. *fukuokaensis*. *Appl. Environ. Microbiol*. **1997**, *63*, 4664−4670.
(24) Shao, Z.; Liu, Z.; Yu, Z. Effects of the 20-kilodalton helper protein on Cry1Ac production and spore formation in *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **2001**, *67*, 5362−5369.
(25) Wu, D.; Federici, B. A. A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. *J. Bacteriol.* **1993**, *175*, 5276−5280.
(26) Chang, C.; Yu, Y. M.; Dai, S. M.; Law, S. K.; Gill, S. S. High-level *cryIVD* and *cytA* gene expression in *Bacillus thuringiensis* does not require the 20-kilodalton protein, and the coexpressed gene products are synergistic in their toxicity to mosquitoes. *Appl. Environ. Microbiol.* **1993**, *59*, 815−821.
(27) Andrews, R. E. J.; Bibilos, M. M.; Bulla, L. A. Protease activation of entomocidal protoxin of *Bacillus thuringiensis* subsp. *kurstaki*. *Appl. Environ. Microbiol.* **1985**, *50*, 732−742.
(28) Nicholson, W. L.; Park, Y. K.; Henkin, T. M.; Won, M.; Weickert, M. J.; Gaskell, J. A.; Chambliss, G. H. Catabolite repression resistant mutation of the *Bacillus subtilis* α-amylase promoter affect transcription levels and are in an operator-like sequence. *J. Mol. Biol.* **1987**, *198*, 609−618.
(29) Mohan, M.; Gujar, G. T. Susceptibility pattern and development of resistance in the diamondback moth, *Plutella xylostella* L, to *Bacillus thuringiensis* Berl var *kurstaki* in India. *Pest Manage. Sci.* **2000**, *56*, 189−194.
(30) Damgaard, P. H.; Granum, P. E.; Bresciani, J.; Torregrossa, M. V.; Eilenberg, J.; Valentino, L. Characterization of *Bacillus thuringiensis* isolated from infections in burn wounds. *FEMS Immunol. Med. Microbiol.* **1997**, *18*, 47−53.

Received for review July 31, 2002. Revised manuscript received October 3, 2002. Accepted October 3, 2002. This work was supported by the National Science Council, Taipei, Taiwan. Project No. NSC-89-2313-B005-309 and NSC-90-2313-B005-149.

JF025863L