J. Microbiol. Biotechnol. (2007), **17**(1), 15–20

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Identification and Molecular Characterization of Novel *cry1*-Type Toxin Genes from *Bacillus thuringiensis* K1 Isolated in Korea

LI, MING SHUN[1], JAE YOUNG CHOI[2], JONG YUL ROH[3], HEE JIN SHIM[3], JOONG NAM KANG[3], YANG-SU KIM[3], YONG WANG[3], ZI NIU YU[1], BYUNG RAE JIN[4], AND YEON HO JE[3]*

[1]*State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Lion Street, Hongshan District, Wuhan 430070, China*
[2]*Research Institute for Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Korea*
[3]*School of Agricultural Biotechnology, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Korea*
[4]*Division of Biotechnology, College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Korea*

Received: June 7, 2006
Accepted: August 14, 2006

Abstract To clone novel *cry1*-type genes from the *Bacillus thuringiensis* K1 isolate, about 2.4-kb-long PCR fragments were amplified with two primer sets of ATG1-F/N400-R and 1BeATG1-F/N400-R. Using PCR-RFLP, three novel *cry1*-type genes, *cry1-1*, *cry1-7*, and *cry1-44*, were obtained from *B. thuringiensis* K1 and the complete coding sequences of these novel genes were analyzed. The Cry1-1, Cry1-7, and Cry1-44 proteins showed maximum similarities of about 78.0%, 99.7%, and 91.0% with the Cry1Ha1, Cry1Be1, and Cry1Ac2 proteins, respectively. These novel *cry1*-type genes were expressed using a baculovirus expression vector system and their insecticidal activities were investigated. Whereas all three novel genes were toxic to *Plutella xylostella* larvae, only Cry1-1 showed insecticidal activity against *Spodoptera exigua* larvae.

Key words: *Bacillus thuringiensis* K1, RFLP, *cry1*-type gene, inverse PCR, insecticidal activity, baculovirus expression vector system

Bacillus thuringiensis, a Gram-positive and spore-forming soil bacterium, is the most well-known and widely used bioinsecticide for insect pest control. *B. thuringiensis* produces parasporal inclusions during sporulation. The parasporal inclusion is solubilized and cleaved as an active toxin by proteolysis in the insect midgut, and the active toxin interacts with the larval midgut epithelium, causing a disruption in membrane integrity and ultimately leading to insect death [8]. The crystal proteins are mainly toxic to the larvae of lepidopteran, dipteran, and coleopteran insects [13, 19]. In addition, they are toxic to some hymenoptera, homoptera, and mallophaga as well as many nematodes, flatworms, and Sarcomastigophora [9, 32]. Since the first cloning of an insecticidal crystal protein gene from *B. thuringiensis* [27], more than 130 crystal proteins based on crystal protein gene sequences and amino acid similarities have been described [1, 5].

However, the conventional use of *B. thuringiensis* insecticides is faced with some limitations, such as a narrow host spectrum, a short shelf life, and development of pest insect resistance. As a solution to overcome these problems, many researchers have concentrated on isolating novel *B. thuringiensis* strains containing high insecticidal activity and/or a wide host spectrum [10, 15, 29]. The need for novel crystal proteins has led to the collection of numerous *B. thuringiensis* isolates and prompted the development of molecular approaches to quickly and easily characterize toxin genes present in *B. thuringiensis* isolates.

For the last few years, PCR-based methods that allowed for both the identification of known *cry* genes and the detection of novel *cry* genes from *B. thuringiensis* isolates have been proposed and broadly applied [1, 4, 21]. However, the PCR-based methods had a major limitation in that they could not isolate the *N*-terminal region of *cry* genes encoding the toxic fragment. Therefore, in this study, we designed two primer sets that enable to detect the active region of all possible *cry1*-type genes, and identified novel *cry1*-type genes from the *B. thuringiensis* K1 isolate using these primers.

**Corresponding author*
Phone: 82-2-880-4706; Fax: 82-2-878-4706;
E-mail: btrus@snu.ac.kr

Materials and Methods

Bacterial Strains and Culture Media

B. thuringiensis isolates were obtained from soil samples collected in Korea using the method of Ohba and Aizawa [24]. *B. thuringiensis* subsp. *kurstaki* HD-1 and *aizawai* HD133 strains, which were kindly provided by Dr. Ohba (Institute of Biological Control, Faculty of Agriculture, Kyushu University, Japan), were used as reference *B. thuringiensis* strains. SPY medium was used for the preparation of plasmid DNA [28].

PCR, Cloning, and RFLP Analysis

Plasmid DNAs of *B. thuringiensis* strains were prepared using a Qiagen Plasmid Midi Kit (Qiagen Co., Germany) according to the manufacturer's protocols. The ATG1-F (5'-ATGCAATGCGTACCTTACAATTGTTTAAGTAAT-3') and N400-R (5'-CATCGATTCGGTTCACCGCACCTTCC-3') primers were designed to isolate and characterize the *cry1*-type genes except *cry1B*-type genes. The 1BeATG1-F primer (5'-ATGACTTCAAATAGGAAAAATGAG-3') was specially designed for the amplification of *cry1B*-type genes because they have a shifted open reading frame at the active region compared with other *cry1*-type genes. The PCR reaction was conducted with 50 ng of plasmid DNA, 20 pM of each primer, and 5 unit of Pyrobest DNA polymerase (Takara Shuzo Co., Japan) in 100 µl of PCR reaction mixture for 30 PCR thermal cycles (94°C for 1 min, 47°C for 30 s, and 72°C for 4 min). Amplification was accomplished with the DNA Thermal Cycler (Perkin Elmer Cetus, U.S.A.). About 2.4 kb of PCR-amplified fragments were purified with a QIAquick PCR Purification Kit (Qiagen Co., Germany) and cloned into the pGem-T Easy vector (Promega Co., U.S.A.). For search of novel *cry1*-type genes, the cloned *cry1*-type genes were digested with EcoRI, EcoRI/HindIII, and EcoRI/EcoRV (Roche Applied Science, Germany). Restriction endonuclease patterns were analyzed on a 2% Nusive 3:1 agarose gel (BMA Co., U.S.A.).

Inverse-PCR and Sequencing

Five µg of plasmid DNA was digested with NdeI or EcoRV (Roche Co., Germany), and extracted twice with phenol, once with chloroform, and dissolved in TE (pH 8.0) at a concentration of 100 µg/ml. One ml of restriction fragments was diluted to a concentration of 0.5 µg/ml with the ligation buffer (Promega Co., U.S.A.). The ligation reaction was initiated by the addition of T4 DNA ligase (Promega Co., U.S.A.) and allowed to proceed for 16 h at 4°C. The ligated samples were then treated with an equal volume of the phenol:chloroform mixture, the aqueous phase was harvested, and the DNA was precipitated with ethanol and collected by centrifugation. These NdeI- or EcoRV-digested and circularized plasmid DNAs were used as the template in inverse PCR [16]. These inverse PCR-amplified fragments were cloned into the pGem-T Easy vector (Promega Co., U.S.A.). The nucleotide sequences were determined by Macrogen Co. (Korea).

Expression of Cry1-Type Crystal Proteins

About 2.4 kb of PCR-amplified toxic fragments of the *cry1*-type genes were inserted into pOB I vector under the control of a polyhedrin gene promoter to construct transfer vectors. One µg of bApGOZA DNA and 4 µg of each transfer vector DNA were cotransfected into Sf9 cells using Cellfectin reagent (Invitrogen Co., U.S.A.) as previously described [17]. The virus-containing supernatant was harvested at 5 days post-transfection, and recombinant AcMNPVs were isolated using plaque purification [25].

SDS PAGE

Sf9 cells infected with the recombinant viruses were lysed with the cell-lysis buffer (50 mM Tris-HCl, pH 8.0, 0.4% SDS, 10 mM EDTA, 5% β-mercaptoethanol), washed twice with the excess phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3), and analyzed in 12% SDS-PAGE.

To confirm the activation of Cry1-type proteins, gut juice from *Bombyx mori* was treated using a partially modified method of Pietrantonio and Gill [26]. The polyhedra produced by recombinant viruses were solubilized in the sodium carbonate solution (0.1 M Na_2CO_3, 0.17 M NaCl, 0.01 M EDTA, pH 10.8) at 37°C for 1 h. The insoluble materials were removed by centrifugation at 10,000 ×*g* for 10 min at 4°C. The supernatant fraction was then incubated with *B. mori* gut juice (2:1 v/v) at 37°C for 2 h. Samples treated with the gut juice were subjected to 10% SDS-PAGE.

Bioassays

The toxicities of recombinant polyhedra containing the Cry1-type crystal proteins were determined against the larvae of *Plutella xylostella* and *Spodoptera exigua.* The purified recombinant polyhedra samples were subjected to 10% SDS-PAGE and the fusion protein content was determined by the 1D-gel analysis system (Kodak Co., U.S.A.). To determine the median lethal concentration (LC_{50}) against *P. xylostella*, serial dilutions of the recombinant polyhedra (corresponding to 500, 125, 12.5, 1.25, 0.12 ng/ml in terms of crystal protein) were treated on Chinese cabbage leaf pieces (2×2 cm^2) and fifteen 2nd-instar larvae were introduced to each leaf surface. To determine the insecticidal activity against *S. exigua,* serial dilutions of the recombinant polyhedra (corresponding to 2,000, 1,000, 500, 125, and 31 ng/ml in terms of crystal protein) were tested on Chinese cabbage leaf pieces onto which fifteen 1st-instar larvae were introduced. All tests were repeated 3 times. The mortality was checked every 12 h for 2 days for

P. xylostella, and every 12 h for 3 days for *S. exigua*. LC_{50} was calculated using Probit analysis [7].

RESULTS

Identification of Novel *cry1*-Type Toxin Genes

In order to search novel *cry1*-type crystal protein genes toxic to lepidopteran insects from the *B. thuringiensis* K1 isolate, we carried out PCR amplification of *cry1*-type genes using the two primer sets of ATG1-F/N400-R and 1BeATG1-F/N400-R. These primers were designed to probe the most conserved regions of all known *cry1*-type gene sequences so that the amplified PCR fragments templated from *B. thuringiensis* strains may contain all possible active regions of *cry1*-type genes. Furthermore, transcription start codon ATG was added to the 5' end of sense primers, ATG1-F and 1BeATG1-F, for the direct application of PCR-amplified fragments in the protein expression study. About 2.4-kb fragments including active regions of the *cry1*-type genes were amplified from *B. thuringiensis* K1 using these primers (Fig. 1A). These PCR fragments were cloned into the pGem-T Easy vector, and five *cry1*-type genes were isolated through RFLP analysis (Fig. 1B). Nucleotide sequence determination of these five *cry1*-type genes revealed that two genes were identical to *cry1Ea* (GenBank Accession No. U94323) and *cry1Aa* (GenBank Accession No. D17518) genes, respectively. However, the other three genes, *cry1-1*, *cry1-7*, and *cry1-44*, were different from all of the *cry1*-type genes previously reported.

For analysis of the complete nucleotide sequences of these novel genes, full-length coding regions of *cry1-7* and *cry1-44* were cloned using inverse PCR. On the other hand, to clone the *cry1-1* full-length gene, about 8.0 kb of a HindIII/XhoI-digested restriction fragment was inserted into the pBluescript II SK vector. The *cry1-7* full-length gene (GenBank Accession No. AY570735) was 3,684 bp long, encoding 1,228 amino acid residues. The gene showed a high level of similarities in nucleotide (99.4%) and deduced amino acid (99.3%) sequences with the *cry1Be* gene. Furthermore, the full-length sequence of *cry1-44*

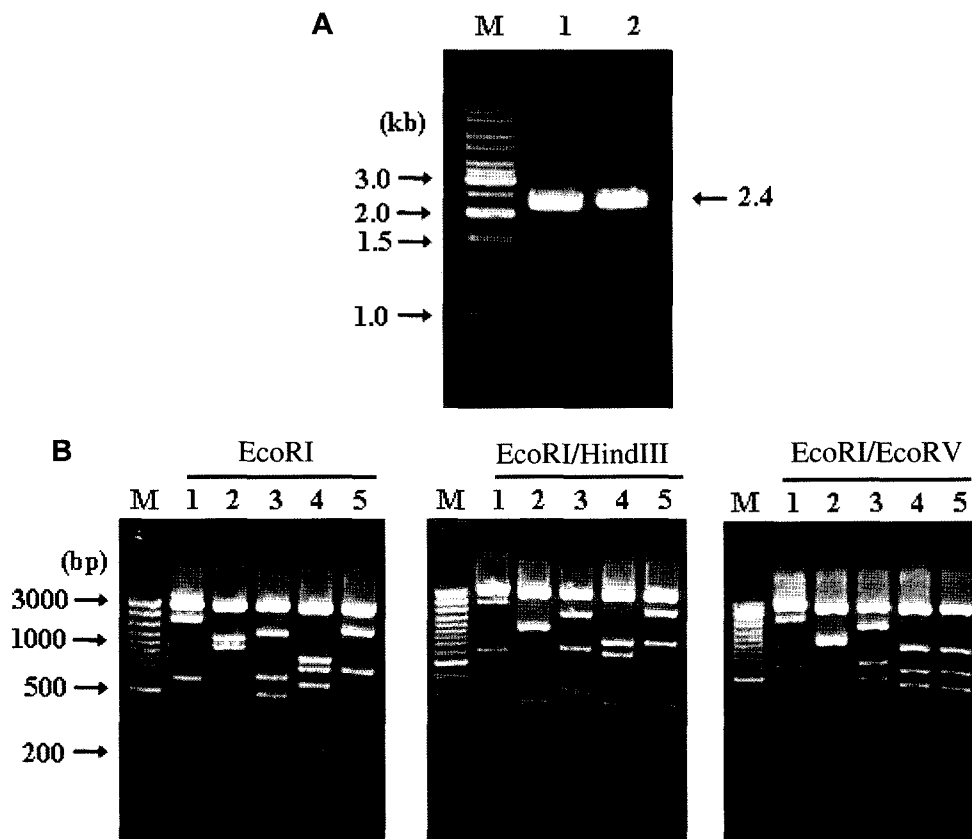

Fig. 1. PCR amplification with the two primer sets, ATG1-F/N400-R and 1BeATG1-F/N400-R (**A**), and RFLP pattern of the *cry1*-type genes (**B**) from *B. thuringiensis* K1.

A. Lanes: M, 1-kb DNA ladder (Fermentas, U.S.A.); 1, PCR amplification with ATG1-F and N400-R primers; 2, PCR amplification with 1BeATG1-F and N400-R primers. **B.** About 2.4 kb of PCR-amplified fragments were cloned into pGem-T easy vector and digested with EcoRI, EcoRI/HindIII, and EcoRI/EcoRV and electrophoresed on a 3% agarose gel. Lanes: M, 100-bp DNA ladder (Fermentas, USA); 1, *cry1-1*; 2, *cry1Ea*; 3, *cry1-7*; 4, *cry1Aa*; 5, *cry1-44*.

(GenBank Accession No. AY570733) was 3,546 bp long, encoding 1,182 amino acid residues. The gene showed 91.2% of nucleotide sequence similarity and 90.1% of amino acid sequence similarity with the *cry1Ac* gene. The *cry1-1* gene (GenBank Accession No. AY554171) was 3,512 bp long, encoding 1,171 amino acid residues and showed maximum similarities in nucleotide (77.6%) and deduced amino acid (73.0%) sequences with the *cryHa* gene.

Insecticidal Activities of Novel Cry1-Type Crystal Proteins

To evaluate the insecticidal activities of novel *cry1*-type genes from *B. thuringiensis* K1, *N*-terminal active regions of these genes as well as Cry1Ac and Cry1C as positive control were expressed using the recombinant polyhedra technique as previously described [3]. In this method, each crystal protein was expressed as a fusion protein with the polyhedrin of AcNPV, and the fusion protein was occluded into polyhedra of AcNPV.

In order to examine the expression of fusion proteins in the polyhedra produced by each recombinant virus, the protein profiles of recombinant viruses were analyzed by SDS-PAGE. Whereas the polyhedra of wtAcMNPV solely consisted of an about 30-kDa polyhedrin protein, about 120–130 kDa of fusion protein along with the 30 kDa of polyhedrin were exhibited in recombinant viruses (Fig. 2A). In addition, recombinant viruses, ApAc and Ap1C, showed about 100 kDa of fusion protein in addition to the polyhedrin protein. To verify whether or not fusion proteins occluded in polyhedra could be cleaved into the active toxin by proteolytic enzymes present in the gut juice, the purified recombinant polyhedra were treated with *B. mori* gut juice. About 65 kDa of the activated Cry1-type protein was exhibited when the recombinant polyhedra were treated with *B. mori* gut juice (Fig. 2B).

Table 1. Median lethal concentration (LC_{50}) of Cry1-type proteins contained in *B. thuringiensis* K1 against *P. xylostella* and *S. exigua* larvae.

	P. xylostella		*S. exigua*	
	LC_{50} (ng/ml)	95% fiducial limits (ng/ml)	LC_{50} (ng/ml)	95% fiducial limits (ng/ml)
Cry1Ac	20.8	10.5–39.4	>2,000	–
Cry1C	>500	–	434.9	373.1–532.1
Cry1Aa	26.7	0.93–29.97	>2,000	–
Cry1Ea	>500	–	667.2	525.8–766.7
Cry1-1	95.1	39.9–293.8	1,105	932.8–1203.0
Cry1-7	199.6	137.0–220.8	>2,000	–
Cry1-44	25.9	11.29–58.08	>2,000	–

The bioassay against *P. xylostella* and *S. exigua* larvae was performed using recombinant polyhedra containing Cry1-type crystal proteins to evaluate the insecticidal activities of the *cry1*-type genes. In the bioassay against *P. xylostella* larvae, the Cry1-44 showed similar LC_{50} values with that of the Cry1Ac (Table 1). The other two proteins, Cry1-1 and Cry1-7, also showed toxicities against *P. xylostella* larvae, although the overall LC_{50} values of these proteins were higher than that of Cry1Ac. Against *S. exigua* larvae, only Cry1-1 showed toxicity, by a 2.5-fold higher LC_{50} value than that of Cry1C (Table 1). However, the other proteins did not show any activity against *S. exigua* larvae.

Discussion

We have described the biochemical and molecular biological characteristics of a new *B. thuringiensis* K1, which was isolated from a Korean soil sample [22]. In the PCR analysis

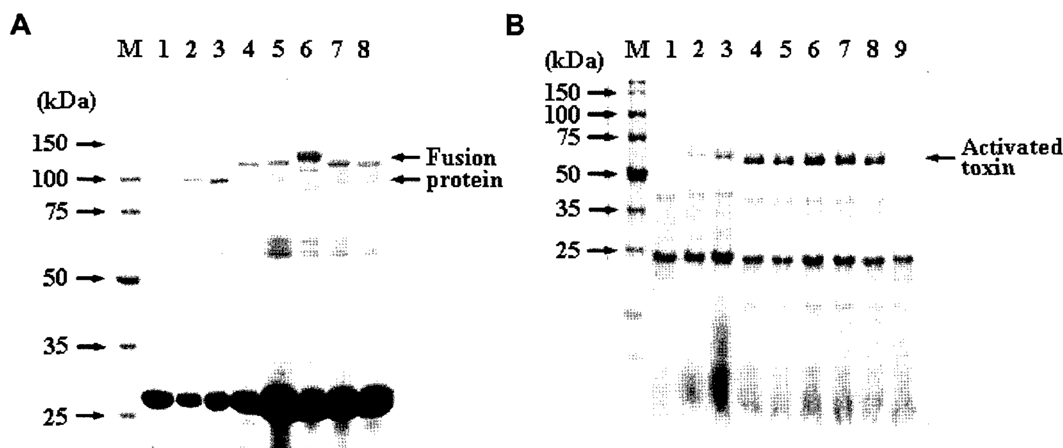

Fig. 2. SDS-polyacrylamide gel electrophoresis (**A**) and activation (**B**) analysis of the polyhedrin-crystal toxin fusion proteins occluded in recombinant polyhedra.

Lanes: M, protein molecular weight marker; 1, wtAcNPV; 2, Cry1Ac; 3, Cry1C; 4, Cry1-1; 5, Cry1Ea; 6, Cry1-7; 7, Cry1Aa; 8, Cry1-44; 9, gut juice of *B. mori*.

with specific primers, this isolate contained five *cry1*-type genes: *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1E*, and *cryX* [22]. In this study, the active regions of the five *cry1*-type genes were identified. Among the five *cry1*-type genes, three genes were revealed to be novel and the other two genes were identical to be the previously reported *cry1Aa* and *cry1Ea* genes [19, 30]. One novel gene, *cry1-44*, showed 91.2% nucleotide sequence similarity with the *cry1Ac* gene; however, the 487-bp specific PCR-amplified product region was identical to the corresponding region of the *cry1Ac* gene. Another novel gene, *cry1-1*, contained a 365 bp PCR product of the *cryX* gene. However, the *cry1-7* gene was firstly isolated using the 1BeATG1-F/N400-R primer set. In this study, we could not isolate the *cry1Ab* gene that was identified in a previous study using a *cry1Ab*-specific primer. The *cry1Ab* PCR product identified previously might be resulted from other *cry1A*-type genes.

The PCR-RFLP is a simple and convenient method to detect both known and new genes existing in *B. thuringiensis* strains [4, 14, 18, 21]. However, the universal primers used in the previous study had a limitation in that its PCR products could not be directly used for a functional assay, since these primers amplify the domain III and partial C-terminal region. To date, various novel *cry* genes have been expressed in the *B. thuringiensis* $Cry^{-}B$ strain to evaluate the insecticidal activity of these genes [2, 20, 23, 30, 31]. However, expression of novel *cry* genes in *B. thuringiensis* cells is very tedious and time-consuming, in that this approach requires to clone the full-length sequence of about 3.6 kb containing the complete coding region of the target gene. Recently, we have described the generation of recombinant baculoviruses in which the *N*-terminal toxic region of crystal protein is actually incorporated into the polyhedra of baculovirus [3, 11, 12]. This approach allows the crystal protein to be delivered to the gut and killed the insect larvae. Therefore, in order to isolate and characterize the active region of all *cry1*-type genes, universal primer sets of ATG1-F/N400-R and 1BeATG1-F/N400-R were newly designed. As a result, about 2.4 kb of *cry1*-type active regions were isolated from *B. thuringiensis* K1 using these primer sets. It was shown that these primer sets could amplify all possible *cry1*-type active regions from *B. thuringiensis* strains, enabling direct application to functional assay, gene modification, or construction of transgenic crops, *etc*.

The *B. thuringiensis* K1 belonging to subsp. *kurstaki* showed high insecticidal effect against *P. xylostella* and *S. exigua* [22]. Up to now, the specific crystal genes working against *P. xylostella* have been reported to be *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B*, and *cry1C* [6]. The *B. thuringiensis* K1 contains *cry1Aa* and *cry1Ea* genes in addition to three novel insecticidal *cry1*-type genes. Four Cry1-type toxins, Cry1-1, Cry1-7, Cry1-44, and Cry1Aa, from *B. thuringiensis* K1 showed a high and low toxicity against *P. xylostella*. Among *B. thuringiensis* crystal protein genes, the *cry1C*, *cry1D*, *cry1E*, and *cry1F* genes showed a high activity against *Spodoptera* species [2, 23, 30]. Among the five crystal proteins expressed from the *B. thuringiensis* K1, the Cry1-1 and Cry1Ea showed insecticidal activities against *S. exigua* larvae. However, LC_{50} values of these two crystal proteins were somewhat lower than that of Cry1C. This result suggested that the high toxicity of *B. thuringiensis* K1 against *S. exigua* might be derived from these two toxin genes or the synergistic effect of the multiple genes.

Acknowledgments

This work was supported by a grant from the BioGreen21 Program, Rural Development Administration, Republic of Korea and the Brain Korea 21 Project.

REFERENCES

1. Ben-Dov, E., A. Zaritsky, E. Dahan, Z. Barak, R. Sinai, R. Manasherob, A. Khamraev, E. Troitskaya, D. Dubitsky, N. Berezina, and Y. Margalith. 1997. Extended screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **63:** 4883–4890.
2. Chambers, J. A., A. Jelen, M. P. Gilbert, C. S. Jany, T. B. Johnson, and C. Gawron-Burke. 1991. Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subsp. *aizawai*. *J. Bacteriol.* **173:** 3966–3976.
3. Chang, J. H., J. Y. Choi, B. R. Jin, J. Y. Roh, J. A. Olszewski, S. J. Seo, D. R. O'Reilly, and Y. H. Je. 2003. An improved baculovirus insecticide producing occlusion bodies that contain *Bacillus thuringiensis* insect toxin. *J. Inverbr. Pathol.* **84:** 30–37.
4. Choi, J. Y., M. S. Li, J. Y. Roh, H. J. Shim, K. S. Boo, and Y. H. Je. 2003. Identification of novel insecticidal *cry1*-type genes from *Bacillus thuringiensis* strains isolated in Korea. *J. Asia-Pacific Entomol.* **6:** 229–233.
5. Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum, and D. H. Dean. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62:** 807–813.
6. Entwistle, F. P., S. J. Cory, J. M. Bailey, and S. Higgs. 1993. *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice*, pp. 9–11. John Wiley and Sons, Chichester, England.
7. Finney, D. J. 1971. *Probit Analysis*, 3rd Ed. Cambridge University Press, U.K.
8. Gill, S. S., E. A. Cowlest, and V. Francis. 1995. Identification, isolation and cloning of a *Bacillus thuringiensis* CryIAc toxin-binding protein from midgut of the lepidopteran insect *Heliothis virescens*. *J. Biol. Chem.* **270:** 27277–27282.
9. Horak, P., J. Weiser, and L. M. Scaron. 1996. The effect of *Bacillus thuringiensis* M-exotoxin of *Trematode cercariae*. *J. Invertebr. Pathol.* **68:** 41–49.

10. Hwang, S. H., H. Saitoh, E. Mizuki, K. Higuchi, and M. Ohba. 1998. A novel class of mosquitocidal δ-endotoxin, Cry19B, encoded by a *Bacillus thuringiensis* serovar *higo* gene. *Syst. Appl. Microbiol.* **21:** 179–184.
11. Je, Y. H., B. R. Jin, H. W. Park, J. Y. Roh, J. H. Chang, and S. K. Kang. 1999. Construction of a novel recombinant baculovirus producing polyhedra with a *Bacillus thuringiensis* Cry1Ac crystal protein. *J. Korean Soc. Virol.* **29:** 145–153.
12. Je, Y. H., B. R. Jin, J. Y. Roh, J. H. Chang, S. J. Seo, J. A. Olszewski, D. R. O'Reilly, and S. K. Kang. 2003. Baculovirus expression vectors that incorporate the foreign protein into viral occlusion bodies. *Biotechniques* **34:** 81–87.
13. Johnson, C., A. H. Bishop, and C. L. Turner. 1998. Isolation and activity of strains of *Bacillus thuringiensis* toxic to larvae of the housefly (Diptera: Muscidae) and tropical blowflies (Diptera: Calliphoridae). *J. Invertebr. Pathol.* **71:** 138–144.
14. Jung, S.-Y., J.-H. Lee, Y.-G. Chai, and S.-J. Kim. 2005. Monitoring of microorganisms added into oil-contaminated microenvironments by terminal-restriction fragment length polymorphism analysis. *J. Microbiol. Biotechnol.* **15:** 1170–1177.
15. Kim, H. S., D. W. Lee, S. D. Woo, Y. M. Yu, and S. K. Kang. 1998. Distribution, serological identification, and PCR analysis of *Bacillus thuringiensis* isolated from soils of Korea. *Curr. Microbiol.* **89:** 16–23.
16. Kim, J.-N., S.-J. Lee, H.-S. Lee, and H.-G. Rhie. 2005. Inactivation of *mutS* leads to a multiple-drug resistance in *Pseudomonas putida* ATCC12633. *J. Microbiol. Biotechnol.* **15:** 1214–1220.
17. Kim, J. S., J. Y. Choi, J. H. Chang, H. J. Shim, J. Y. Roh, B. R. Jin, and Y. H. Je. 2005. Characterization of an improved recombinant baculovirus producing polyhedra that contain *Bacillus thuringiensis* Cry1Ac crystal protein. *J. Microbiol. Biotechnol.* **15:** 710–715.
18. Kim, W.-K., R. C., and D. Jahng. 2005. Enrichment of ammonia-oxidizing bacteria for efficient nitrification of wastewater. *J. Microbiol. Biotechnol.* **15:** 772–779.
19. Kondo, S., N. Tamura, A. Kunitate, M. Hattori, A. Akashi, and I. Ohmori. 1987. Cloning and nucleotide sequencing of two insecticidal delta-endotoxin genes from *Bacillus thuringiensis* var. *kurstaki* HD-1 DNA. *Agric. Biol. Chem.* **51:** 455–463.
20. Koo, B. T., S. H. Park, S. K. Choi, B. S. Shin, J. I. Kim, and J. H. Yu. 1995. Cloning of a novel crystal protein gene *cry1K* from *Bacillus thuringiensis* subsp. *morrisoni*. *FEMS Microbiol. Lett.* **134:** 159–164.
21. Kuo, W. S. and K. F. Chak. 1996. Identification of novel *cry*-type genes from *Bacillus thuringiensis* strains on the basis of restriction fragment length polymorphism of the PCR-amplified DNA. *Appl. Environ. Microbiol.* **62:** 1369–1377.
22. Li, M. S., Y. H. Je, I. H. Lee, J. H. Chang, J. Y. Roh, H. S. Kim, H. W. Oh, and K. S. Boo. 1995. Isolation and characterization of a strain of *Bacillus thuringiensis* ssp. *kurstaki* containing a new δ-endotoxins gene. *Curr. Microbiol.* **45:** 299–302.
23. Moar, W. J., M. Pusztai-Carey, H. Van Faassen, D. Bosch, R. Frutos, C. Rang, K. Luo, and M. J. Adang. 1995. Development of *Bacillus thuringiensis* Cry1C resistance by *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae). *Appl. Environ. Microbiol.* **61:** 2086–2092.
24. Ohba, M. and K. Aizawa. 1978. Serological identification of *Bacillus thuringiensis* and related bacteria isolated in Japan. *J. Invertebr. Pathol.* **32:** 303–309.
25. O'Reilly, D. R., L. K. Miller, and V. A. Luckow. 1992. *Baculovirus Expression Vectors: A Laboratory Manual.* Oxford University Press, New York.
26. Pietrantonio, P. V. and S. S. Gill. 1992. The parasporal inclusion of *Bacillus thuringiensis* subp. *shandongiensis*: Characterization and screening for insecticidal activity. *J. Invertebr. Pathol.* **59:** 295–302.
27. Schnepf, H. E. and H. R. Whiteley. 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **78:** 2893–2897.
28. Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proc. Natl. Acad. Sci. USA* **44:**1072–1078.
29. Tounsi, S., A. J'Mal, N. Zouari, and S. Jaoua. 1999. Cloning and nucleotide sequence of a novel *cry1Aa*-type gene from *Bacillus thuringiensis* subsp. *kurstaki*. *Biotechnol. Lett.* **21:** 771–775.
30. Visser, B., E. Munsterman, A. Stocker, and W. G. Dirkse. 1990. A novel *Bacillus thuringiensis* gene encoding a *Spodoptera exigua*-specific crystal protein. *J. Bacteriol.* **172:** 6783–6788.
31. Von Tersch, M. A., H. L. Robbins, C. S. Jany, and T. B. Johnson. 1991. Insecticidal toxins from *Bacillus thuringiensis* subsp. *kenyae*: Gene cloning and characterization and comparison with *B. thuringiensis* subsp. *kurstaki* Cry1A(c) toxins. *Appl. Environ. Microbiol.* **57:** 349–358.
32. Walters, F. S. and L. H. English. 1995. Isolation and characterization of a strain of *Bacillus thuringiensis* δ-endotoxins toward the potato aphid in an artificial diet bioassay. *Entomol. Exp. Appl.* **77:** 211–216.