Int. J. Indust. Entomol. Vol. 3, No. 1, 2001, pp. 19~23

International Journal of
Industrial Entomology

Isolation and Characterization of Two Mosquitocidal *Bacillus thuringiensis* Strains Belonging to subsp. *kurstaki* and subsp. *aizawai*

Jong Yul Roh, Ming Shun Li, Jin Hee Chang, Hee Jin Shim, Byung Rae Jin[1] and Yeon Ho Je*

School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea.
[1] College of Natural Resources and Life Science, Dong-A University, Pusan 604-714, Korea.

(Received 15 June 2001; Accepted 15 July 2001)

Two *B. thuringiensis* strains, which possess mosquitocidal activities, were isolated from Korean soil samples and named K-1205-1 and K-1381-1. Serological studies indicated that K-1205-1 and K-1381-1 belonged to *B. thuringiensis* subsp. *kurstaki* (H3a3b3c) and subsp. *aizawai* (H7), respectively. K-1205-1 produced typical bipyramidal parasporal inclusions, but K-1381-1 produced irregular bipyramidal shape. Total plasmid DNA patterns analysis showed that K-1205-1 and K-1381-1 were different from their reference strains, subsp. *kurstaki* and subsp. *aizawai*, respectively, in high molecules, whereas their crystal protein patterns showed no difference. The *cry* gene contents of K-1205-1 and K-1381-1 were identical with those of the reference strains. Mosquitocidal activities of crystal proteins produced by K-1205-1 and K-1381-1 were significantly high by about 40-50 folds at LC_{50} when compared to those of subsp. *kurstaki* and subsp. *aizawai*. Finally, in southern blot analysis using *cry1A*- type specific probe, K-1205-1 and K-1381-1 had different bands from subsp. *kurstaki* and subsp. *aizawai*, respectively. In conclusion, our results suggest that K-1205-1 and K-1381-1 appear to be new moquitocidal *B. thuringiensis* strains isolated from Korean soil.

Key words : *Bacillus thuringiensis* subsp. *kurstaki*, subsp. aizawai, K-1205-1, K-1381-1, *Culex pipiens*, mosquitocidal activities

*To whom correspondence should be addressed.
School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea. Tel: 82-31-290-2492; Fax: 82-31-296-0926; E-mail: btrus@hanmail.net

Introduction

Many *Bacillus thuringiensis* variants have been isolated from natural environments such as soil, silkworm litter, animal feed mill and the phylloplane of deciduous and conifer trees (Martin and Travers, 1989; Meadows *et al.*, 1992; Smith and Couche, 1991). In Korea, a large number of *B. thuringiensis* strains have been isolated from many habitats, including soil, sericultural farms and stored-product dust (Kim *et al.*, 1998b, 1998c, 1998a). Insecticidal crystal proteins produced by *B. thuringiensis* strains, had high toxicities against lepidopteran, dipteran, and coleopteran larvae (Höfte and Whiteley, 1989). Furthermore, Garcia-Robles *et al.* (2001) reported that a *B. thuringiensis* strain toxic against important hymenopteran forest pests, pine sawfly (*Diprion pini*) and spruce web-spinning sawfly (*Cephacia abietis*).

In many *B. thuringiensis* isolates, the rate of mosquitocidal strains is significantly lower than that of lepidoptera specific strains though mosquito-specific *B. thuringiensis* strains are important in sanitary fields (Martin and Travers, 1989). Besides, only 21 mosquitocidal strains out of total 309 *B. thuringiensis* isolates were found in Korea (Kim *et al.*, 1998b; 1998c; 1998a). In this study, to isolate *B. thuringiensis* strains with high mosquitocidal activities, we investigated two strains belonging to *B. thuringiensis* subsp. *kurstaki* and subsp. *aizawai*, which had been isolated from Korean soil samples.

Materials and Methods

Bacterial strains and growth media

B. thuringiensis colonies were isolated from Korean soil samples as described previously (Kim *et al.*, 1993). *B. thuringiensis* strains, used as references (subsp. *kurstaki* HD-1 and subsp. *aizawai)* and as positive control (subsp.

israelensis) were kindly provided by Dr. M. Ohba (Institute of Biological Control, Faculty of Agriculture, Kyushu University, Japan). *B. thuringiensis* cultures were grown at 30°C with vigorous shaking in SPY (Kronstad *et al.*, 1983) for plasmid preparation and GYS (Nickerson *et al.*, 1974) for production of crystal proteins.

Preparation of H antisera and serological tests

H-antigens and the corresponding antisera were prepared from the type strains of serotypes H1 to H27 (Ohba and Aizawa, 1978). H antisera-antigens agglutination studies were performed using 96 well plates (Laurent *et al.*, 1996).

Morphological observation and SDS-PAGE

B. thuringiensis strains were grown in GYS media for 4 days and the morphology of parasporal inclusions was observed during sporulation period by the phase contrast microscopy. The spore-crystal mixtures after autolysis were harvested, washed 3 times with the washing solution (0.5 M NaCl, 2 mM EDTA) and resuspended in distilled water.

The harvested spore-crystal mixtures of *B. thuringiensis* strains were sonicated three times (22,000 cycle/sec for 1 min) and mixed with the equal volume of 5 x sample buffer [60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.1% bromophenol blue]. Each sample, which was boiled for 5 min, was loaded onto SDS-10% polyacrylamide separating gel with a 3% stacking gel as described by Laemmli (1970). The gel was stained with Coomassie brilliant blue (Sigma Co., USA) and 10 kDa protein ladder (Gibco Co., USA) was used as the standard.

Plasmid DNA isolation and southern blot analysis

The plasmid DNAs of *B. thuringiensis* strains were isolated according to the manufacturer's protocols of Qiagen midi prep. kit (Qiagen Co., Germany). In hybridization experiment for the localization of the *cryIA*-type crystal protein genes, plasmid DNAs purified from *B. thuringiensis* strains were digested with *Hin*dIII and *Xba*I and electrophoresed on a 0.8% agarose gel. The gel was treated for 15 min in 0.25 N HCl and transferred to Hybond N^+ filters (Amersham Co., UK) in 0.4 N NaOH as a transfer buffer. The 740 bp *cryIAa* PCR product was used as a probe. The probe included the conserved region of *cryIA*-type genes family and amplified from subsp. *kurstaki* HD-1, and labeled using the DIG labelling Kit (Amersham Co., UK). Prehybridization, hybridization, washing and detection procedures were followed as described by the manufactures manuals. The signal was visualized by using Hyperfilm-ECL (Amersham Co., UK) exposed to the blot and reacted with the developer and fixer (Kodak Co., USA).

Oligonucleotides and PCR

Twenty primers (cryIAa, IAb, IAc, IB, IC, ID, IE, IF, IG, cryIIA, cryIIIA, IIIB, IIIC, IIID, cryIVA, IVB, IVC, IVD, cryV, and cytA) for the specific *cry* genes used in the PCR analysis were synthesized as reported by Carozzi *et al.* (1991), Gleave *et al.* (1993), Ceron *et al.* (1994; 1995) and Kalman *et al.* (1993). Plasmid DNAs of *B. thuringiensis* strains were used as templates. The reaction was conducted for 250 ng of sample DNA with 2.5 U of *Taq* DNA polymerase (Promega Co., USA), 200 nM each deoxynucleotide triphosphate, 100 pM each primer and 3 mM $MgCl_2$ in a final volume of 50 μl. Amplification was accomplished with the DNA Thermal Cycler (Perkin Elmer Cetus, USA) by using the Step-Cycle program set to denature at 94°C for 1 min, anneal at specific annealing temperature according to each primer set for 1 min, and extend at 72°C for 1 or 2 min, followed by a 4-s-per cycle extension for a total of 35 cycles. PCR products were analyzed by 1.4% agarose gel electrophoresis. The purified PCR products were analyzed by dye termination method in ABI 377 automated sequencer (Applied Biosystems, USA) as specified by the manufacturer.

Bioassay on *Culex pipiens* larvae

Bioassay was performed on the 3-days larvae of *Culex pipiens* with spore-crystal mixtures. Tested larvae were reared at 25°C and 60% humidity. In quantitative bioassay against *C. pipiens*, 20 larvae per each dilution were placed into individual wells of 24-well plate (Falcon Co., USA) containing 1 ml diluted inoculation solutions with distilled water and each treatment was repeated 3 times. Mortality was checked by counting the dead larvae after 24 hrs and median lethal concentration was calculated using Probit analysis (Russell *et al.*, 1977).

Results and Discussion

To isolate mosquitocidal *B. thuringiensis* strains, many *B. thuringiensis* isolates from Korean soil samples were bioassayed against *Culex pipiens* larvae. Through the qualitative bioassay, two *B. thuringiensis* strains, having relatively high mosquitocidal activities, were selected and named K-1205-1 and K-1381-1. Serological studies indicated that K-1205-1 and K-1381-1 seemed to be subsp. *kurstaki* (H3a3b3c) and subsp. *aizawai* (H7), respectively. K-1205-1 produced typical bipyramidal parasporal inclusions and no significant difference was found in the shapes and sizes of vegitative cells, spores, and parasporal inclusions from subsp. *kurstaki* HD-1. But the parasporal

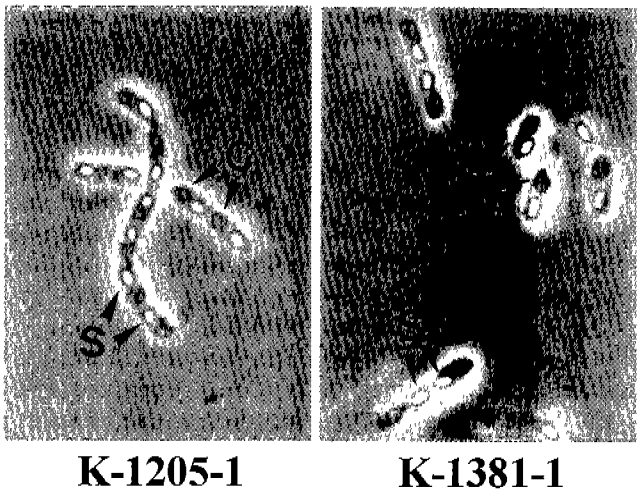

Fig. 1. Phase-contrast micrographs of sporulated cells of *B. thuringiensis* K-1205-1 and K-1381-1. S and C indicate spore and crystal protein, respectively.

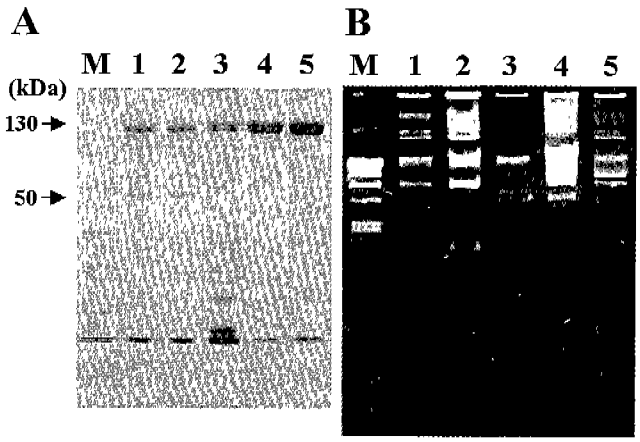

Fig. 2. SDS-PAGE of parasporal inclusions (A) and total plasmid DNA pattern (B) of *B. thirngiensis* strains. Panel A: M, 10 kDa protein ladder (Gibco BRL); 1, subsp. *kurstaki* HD-1; 2, K-1205-1; 3, subsp. *israelensis*; 4, K-1381-1; 5, subsp. *aizawai*. Panel B: M, lambda DNA digested with *Hin*dIII; 1, 2, 3, 4 and 5 are identical with a Panel A.

inclusions of K-1381-1 were irregular bipyramidal shape, being composed of elongated proximal part, and relatively bigger (1-1.9 μm) than those of subsp. *aizawai* (Fig. 1).

Crystal protein components of K-1205-1 and K1381-1 were similar to those of subsp. *kurstaki* HD-1 and subsp. *aizawai*, respectively, but quite different from those of subsp. *israelensis* (Fig. 2A). Total plasmid DNA analysis showed that K-1205-1 and K-1381-1 were different from their reference strains in high molecules. But K-1205-1 had the typical small plasmid of subsp. *kurstaki* HD-1 (Fig. 2B). For detection of *cry* genes of two isolates, PCR analysis was performed using *cry* gene-specific primers. The control strains, *B. thuringiensis* subsp. *kurstaki* HD-1 showed products of *cry1Aa, cry1Ab, cry1Ac* and *cry2*, and subsp. *aizawai* showed *cry1Aa, cry1Ab,* and *cry1C* genes (Höfte and Whiteley, 1989). The *cry* gene contents of K-1205-1 and K-1381-1 were identical with those of their reference strains (data not shown).

Mosquitocidal activities of crystal proteins produced by K-1205-1 and K-1381-1 were evaluated against *C. pipiens* and compared with those of subsp. *kurstaki* and subsp. *aizawai* as well as that of the positive control, subsp. *israelensis* (Table 1). In qualitative bioassay, two strains

Table 1. Toxicities of two *Bacillus thuringiensis* isolates from Korean soil samples against *Culex pipiens*

Strain	n[a]	LC_{50}[b]	(FL_{95})[b]	Slope
ISR[c]	180	0.32	(0.05-0.84)	0.52 ± 0.13
1205-1	240	21.88	(12.61-35.50)	2.41 ± 0.39
1381-1	240	17.11	(10.88-26.51)	2.43 ± 0.36
KUR[c]	180	> 900		
AIZ[c]	180	ND[d]		

[a] Number of tested insects.
[b] LC_{50} and FL_{95} are expressed as X 10^6 CFU per ml of inoculated solution.
[c] ISR, *B. thuringiensis* subsp. *israelensis*; KUR, subsp. *kurstaki* HD-1; AIZ, subsp. *aizawai*.
[d] ND, not determined.

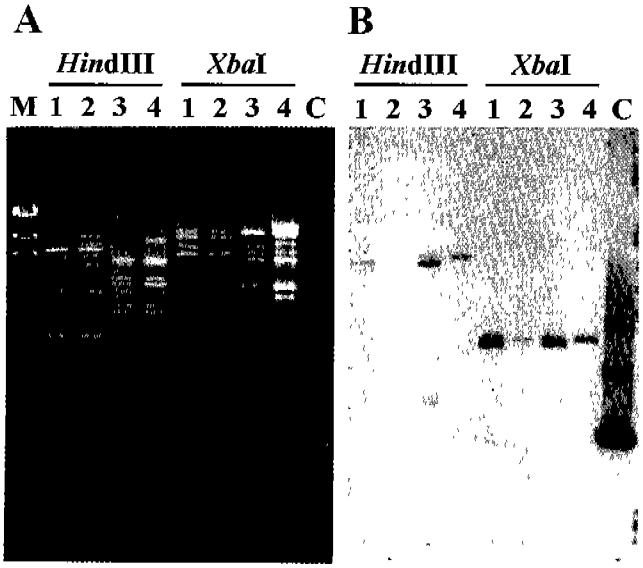

Fig. 3. Electrophoresis (A) and Southern blot analysis (B) of plasmid DNAs digested with *Hin*dIII and *Xba*I of *B. thuringiensis* strains. *Hin*dIII and *Xba*I indicated digestion by each enzyme; M, lambda DNA digested with *Hin*dIII; 1, subsp. *kurstaki* HD-1; 2, K-1205-1; 3, K-1381-1; 4, subsp. *aizawai*; C, the 740 bp *cry1Aa* PCR products as a control.

had 100% mortalities at the concentration of 1×10^9 CFU/ml (colony forming unit per ml) at 48 hrs after treatment (data not shown). The quantitative bioassay showed that the median lethal concentrations (LC_{50}s) of K-1205-1 and K-1381-1 were significantly higher than that of subsp. *israelensis* used as the positive control, however they were much lower by about 40-50 folds when compared to that of subsp. *kurstaki*. Moreover, *B. thuringiensis* subsp. *aizawai* had no mosquitocidal activity. This result confirmed that K-1205-1 and K-1381-1 had significantly higher mosquitocidal activities than subsp. *kurstaki* HD-1 and subsp. *aizawai*.

To investigate the causes of high mosquitocidal activities of K-1205-1 and K-1381-1, we tried to elucidate molecular characteristics of the full *cry* genes in them. Firstly, we compared *cry1A-type* genes localization between two isolates and the reference strains because the genetic informations of the full gene were contributed to the insecticidal spectrum of *B. thuringiensis* strain (Schnepf *et al.*, 1998). In Southern blot analysis using *cry1A*-type specific probe, K-1205-1 had different band from subsp. *kurstaki* and K-1381-1 also had different bands from subsp. *aizawai* (Fig. 3). Unexpectedly, K-1205-1 was similar to subsp. *aizawai* and K-1381-1 to subsp. *kurstaki*.

In conclusion, our results demonstrated that K-1205-1 and K-1381-1 are new *B. thuringiensis* isolates, which had significantly higher mosquitocidal activities as compared with the reference strains. We are now focusing on the molecular characteristics of the full *cry* genes in two strains.

Acknowledgements

This work was supported by the Brain Korea21 Project.

References

Carozzi, N. B., V. C. Kramer, G. W. Warren, S. Ecola and M. G. Koziel (1991) Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. *Appl. Environ. Microbiol.* **57**, 3057-3061.

Ceron, J, L. Covarrubias, R. Quintero, A. Ortiz, M. Ortiz, E. Aranda, L. Linda and A. Bravo (1994) PCR analysis of the *cryI* insecticidal crystal family genes from *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **60**, 353-356.

Ceron, J, A. Ortiz, R. Quintero, L. Guereca and A. Bravo (1995) Specific PCR primers detected to identify *cryI* and *cryIII* genes within a *Bacillus thuringiensis* strain collection. *Appl. Environ. Microbiol.* **61**, 3826-3831.

Garcia-Robles, I., J. Sanchez, A. Gruppe, A. C. Martinez-Ramirez, C. Rausell, M. D. Real and A. Bravo (2001) Mode of action of *Bacillus thuringiensis* PS86Q3 strain in hymenopteran forest pests. *Insect Biochem. Mol. Biol.* **31**, 849-856.

Gleave, A. P., R. Williams and R. J. Hedqes (1993) Screening by polymerase chain reaction of *Bacillus thuringiensis* serotypes for the presence of *cryV*-like insecticidal protein genes and characterization of *cryV* gene cloned from *B. thuringiensis* subsp. *kurstaki*. *Appl. Environ. Microbiol.* **59**, 1683-1687.

Höfte, H. and H. R. Whiteley (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**, 242-255.

Kalman, S., K. L. Kiehne, J. L. Libs and T. Yamamoto (1993) Cloning of a novel *cryIC*-type gene from a strain of *Bacillus thuringiensis* subsp. *galleriae*. *Appl. Environ. Microbiol.* **59**, 1131-1137.

Kim, H. S., H. W. Park, S. H. Kim, Y. M. Yu, S. J. Seo and S. K. Kang (1993) Dual specificity of δ-endotoxin produced by newly isolated *Bacillus thuringiensis* NT0423. *Kor. J. Appl. Entomol.* **32**, 426-432.

Kim, H. S., D. W. Lee, S. D. Woo, Y. M. Yu and S. K. Kang (1998a) Biological, immunological, and genetic analysis of *Bacillus thuringiensis* isolated from granary in Korea. *Curr. Microbiol.* **37**, 52-57.

Kim, H. S., D. W. Lee, S. D. Woo, Y. M. Yu and S. K. Kang (1998b) Distribution, serologica identification, and PCR analysis of *Bacillus thuringiensis* isolated from soils of Korea. *Curr. Microbiol.* **37**, 195-200.

Kim, H. S., D. W. Lee, S. D. Woo, Y. M. Yu and S. K. Kang (1998c) Seasonal distribution and characterization of *Bacillus thuringiensis* isolated from sericultural environments in Korea. *J. Gen. Appl. Microbiol.* **44**, 133-138.

Kronstad, J. W., H. E. Schnepf and H. R. Whiteley. (1983) Diversity of locations for *Bacillus thuringiensis* crystal protein genes. *J. Bacteriol.* **154**, 419-428.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Laurent, P., H. Ripouteau, V. C. Dumanoir, E. Frachon and M. M. Lecadet (1996) A micromethod for serotyping *Bacillus thuringiensis*. *Lett. Appl. Microbiol.* **22**, 259-261.

Martin, P. A. W. and R. S. Travers (1989) Worldwide abundunce and distribution of *Bacillus thuringiensis* isolates. *Appl. Environ. Microbiol.* **55**, 2437-2442.

Meadows, M. P., D. J. Ellis, J. Butt, P. Jarrett and H. D. Burges (1992) Distribution, frequency, and diversity of *Bacillus thuringiensis* in an animal feed mill. *Appl. Environ. Microbiol.* **58**, 1344-1350.

Nickerson, K. W., G. St. Julian and L. A. Bulla, Jr. (1974) Physiology of spore forming bacteria associated with insects: Radiorespirometric survey of carbohydrate metabolism in the 12 serotypes of *Bacillus thuringiensis*. *Appl. Microbiol.* **28**, 129-132.

Ohba, M. and K. Aizawa (1978) Serological identification of *Bacillus thuringiensis* and related bacteria isolated in Japan.

J. Invertbr. Pathol. **32**, 303-309.

Russell, R. M., J. L. Robertson and S. E. Sauvin (1977) POLO: a new computer program for probit anaysis. *Bull. Entomol. Soc. Am.* **23**, 209-213.

Schnepf, E., N. Crickmore, J. V. Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigier and D. H. Dean (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**, 775-806.

Smith, R. A. and G. A. Couche (1991) The phylloplane as a sourse of *Bacillus thuringiensis* variants. *Appl. Environ. Microbiol.* **57**, 311-315.