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Isolation and Characterization of a *Bacillus thuringiensis* ssp. *kurstaki* Strain Toxic to *Spodoptera exigua* and *Culex pipiens*

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Abstract. A strain of *Bacillus thuringiensis* with dual toxicity was isolated from Korean soil samples and named K2. K2 was determined as ssp. *kurstaki* (H3a3b3c) by serological test and produced bipyramidal-shaped parasporal inclusions. The plasmid and protein profiles of *B. thuringiensis* K2 were different from those of the reference strain, ssp. *kurstaki* HD-1. To verify gene type of *B. thuringiensis* K2, PCR analysis with specific *cry* gene primers was performed. The result showed that *B. thuringiensis* K2 had *cry1Aa, cry1Ab, cry1C,* and *cry1D* type genes, whereas ssp. *kurstaki* HD-1 had *cry1Aa, cry1Ab, cry1Ac,* and *cry2* type genes. In addition, *B. thuringiensis* K2 had high toxicity against *Spodoptera exigua* and *Culex pipiens*, whereas *B. thuringiensis* ssp. *kurstaki* HD-1 does not have high toxicity against these two insect species.

Bacillus thuringiensis produces parasporal, proteinaceous, and crystalline inclusions during sporulation. The parasporal inclusion, which may contain more than one type of insecticidal crystal protein, is released with the spore upon lysis of the sporangium [9, 19]. Upon ingestion by the insect, this crystalline inclusion is solubilized in the midgut, releasing proteins called δ-endotoxins. These proteins (protoxins) are activated by midgut protease, and the activated toxins interact with the larval midgut epithelium causing a disruption in membrane integrity and ultimately leading to insect death [7, 19]. Many *B. thuringiensis* strains which have wider spectrums of insecticidal activity express several kinds of crystal protein [19]. In this report, we isolated and characterized a strain of *B. thuringiensis* ssp. *kurstaki* with a wider spectrum to *Spodoptera exigua* and *Culex pipiens*, due to their additional *cry1C* and *cry1D* genes.

Materials and Methods

Bacterial strains and growth media. *B. thuringiensis* colonies were isolated from Korean soil samples by the method of Ohba and Aizawa [15]. *B. thuringiensis* strains used as references, ssp. *kurstaki* HD-1, ssp. *aizawai*, and ssp. *israelensis*, were kindly provided by Dr. M. Ohba (Institute of Biological Control, Faculty of Agriculture, Kyushu University, Japan).

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Preparation of H antisera and H agglutination studies. H-antigens and the corresponding antisera were prepared from the type strains of serotypes H1 to H27 [15]. H antisera-antigens agglutination studies were performed using 96 well plates [14].

Morphological observation and SDS-PAGE. *B. thuringiensis* strains were grown in GYS medium at 28°C until lysis. Spore-crystal mixtures were harvested by centrifugation at 12,000 × g for 20 min, washed three times in washing solution (1M NaCl, 0.01% Tween 20), and resuspended in distilled and autoclaved water. The morphology of parasporal inclusions was observed by phase contrast microscopy and scanning electron microscopy (Phillips SEM 515). The spore-crystal mixture was resuspended in 5× sample buffer (60mM Tris-HCl-(pH 6.8), 25% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.1% bromophenol blue), boiled for 10 min, and subjected to SDS-10% polyacrylamide gel electrophoresis, as described before [5]. SDS-PAGE was performed on a 10% separating gel with a 3% stacking gel as described by Laemmli [13]. The gel was stained with 0.1% Coomassie brilliant blue (Sigma Co., USA).

Oligonucleotides and PCR analysis. 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 δ-endotoxin genes used in the PCR analysis were synthesized as reported by Carozzi *et al.* [2], Gleave *et al.* [6], Ceron *et al.* [3, 4] and Kalman *et al.* [12]. The plasmid DNAs of *B. thuringiensis* strains isolated according to the manufacturer's protocols of Qiagen midi prep. kit (Qiagen Co., Germany) 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 of each deoxynucleotide triphosphate, 100 pM of 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 55°C for 1 min, and extend at 72°C for 1 min, followed by a 4-s-per cycle extension for a total of 35 cycles. Following amplification, the PCR products were ethanol-precipitated, centrifuged at 10,000 × g for 30 min, and rinsed with 70% ethanol. These DNAs were analyzed by 0.9% agarose gel electrophoresis. The purified PCR products were ligated to pGEM-T vector (Promega Co., USA) and analyzed by dye termination method in ABI 377 automated sequencer (Applied Biosystems, USA), as specified by the manufacturer.

Insect bioassays. For primary screening, *B. thuringiensis* isolates were cultured in GYS media until lysis and 1 ml culture were added to the artificial diet with 20 larvae of *Plutella xylostella* or *Spodoptera exigua*, or to 50 ml conical tube containing 10 ml water and 20 larvae of *Culex pipiens*. To estimate the toxicity level of K2, bioassays were conducted at 25°C in 60~70% humidity with a 16L/8D cycle. For quantitative bioassay, 24 of each third instar larvae of *P. xylostella* and the second instar larvae of *S. exigua* were tested with 1 cm × 1 cm × 0.5 cm artificial diet in 24 well plates with various concentrations of *B. thuringiensis* spore-crystal mixtures. Sporulated cultures of *B. thuringiensis* were serially diluted in 0.01% (vol/vol) Triton X-100, and 100 μl aliquots of serial dilutions were applied to the surface of artificial diets. Larval mortality was recorded within three days in *P. xylostella* assay or five days in *S. exigua* assay. In quantitative bioassay against *C. pipiens*, 20 larvae per each dilution were tested. Single late second instar of *C. pipiens* was placed into individual wells of microtiter plate containing 150 μl distilled water and exposed to *B. thuringiensis* suspensions and mortality was checked after three days. All tests were performed with spore-parasporal inclusion suspensions with three replications. Statistical analysis of data was performed with probit anaysis [17].

Results and Discussion

B. thuringiensis K2 was isolated from Korean soil samples for its dual specificity through a primary bioassay. Serological studies indicated that H antigenic structure of the isolate K2 was identical to that of ssp. *kurstaki* (H3a3b3c). *B. thuringiensis* K2 produced typical bipyramidal parasporal inclusions and no significant difference were found in the shapes and sizes of vegitative cells, spores, and parasporal inclusions between K2 and ssp. *kurstaki* HD-1 (data not shown). The plasmid DNA and parasporal inclusion protein patterns of K2 were different from those of ssp. *kurstaki* HD-1. K2 did not have the typical small plasmid of ssp. *kurstaki* HD-1 (Fig. 1). K2 produced one major band with an estimated molecular mass of 130 kDa but did not produce the ca. 65 kDa crystal protein band shown in ssp. *kurstaki* HD-1 (Fig. 2). As seen in Fig. 1, plasmid and protein patterns of K2 were more similar to those of ssp. *aizawai* than those of ssp. *kurstaki* HD-1.

For detection of crystal genes of *B. thuringiensis* strains, PCR analysis was performed using *cry* gene-specific primers (Fig. 3). The control strains, *B. thuringiensis* ssp. *kurstaki* HD-1 showed products of *cry1Aa*, *cry1Ab*, *cry1Ac*, and *cry2*, and ssp. *aizawai* showed *cry1Aa*, *cry1Ab*, and *cry1C* genes [9]. *B. thuringiensis* K2 amplified products of *cry1Aa*, *cry1Ab*, *cry1C*, and *cry1D* genes, different from those of its reference strain, ssp. *kurstaki* HD-1. This *cry* gene content was found in *B. thuringiensis* ssp. *aizawai* strain. The specificity of all PCR products were confirmed by restriction enzyme digestion patterns and DNA sequencing. Sequence analysis suggested that *cry1C*- and *cry1D*-type genes in K2 were identical to *cry1Ca1* (GenBank Accession No. X07518) and *cry1Da1* (GenBank Accession No. X54160), respectively. The *cry1C* gene was isolated from *B. thuringiensis* ssp. *entomocidus* [10] and ssp. *aizawai* [18] and had high specificity against *S. exigua* and *Spodoptera litura*. The *cry1D* gene was isolated from *B. thuringiensis* ssp. *aizawai* HD-68 [8] and was also highly toxic to *S. exigua*. There has been no report of *B. thuringiensis* ssp. *kurstaki* strains encoding CryIC and CryID crystal proteins. Therefore, although K2 was serotyped as ssp. *kurstaki*, it is a new *B. thuringiensis* strain having a different gene type. This unusual multiple gene type should result from conjugation of plasmids or movement of transposable elements between adjacent other cells [22]. The toxin genes are often located on large self-transmissible or mobilizable plasmids [19]. Plasmids p44 and pHT73 harbor genes for insect toxin

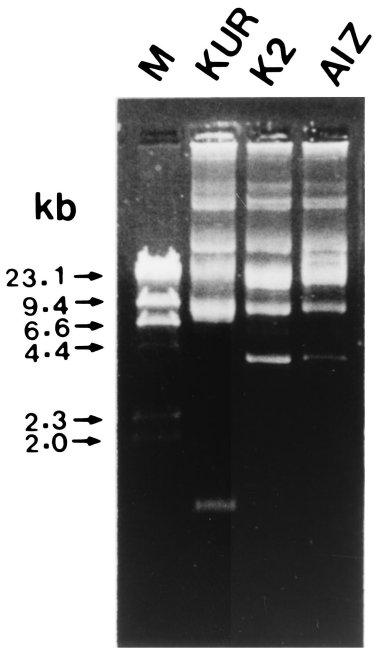

Fig. 1. Plasmid DNA pattern of *B. thuringiensis* strains. Lanes: KUR, *B. thuringiensis* ssp. *kurstaki* HD-1; K2, K2; AIZ, ssp. *aizawai*. M indicates Lambda DNA digested with *Hin*dIII.

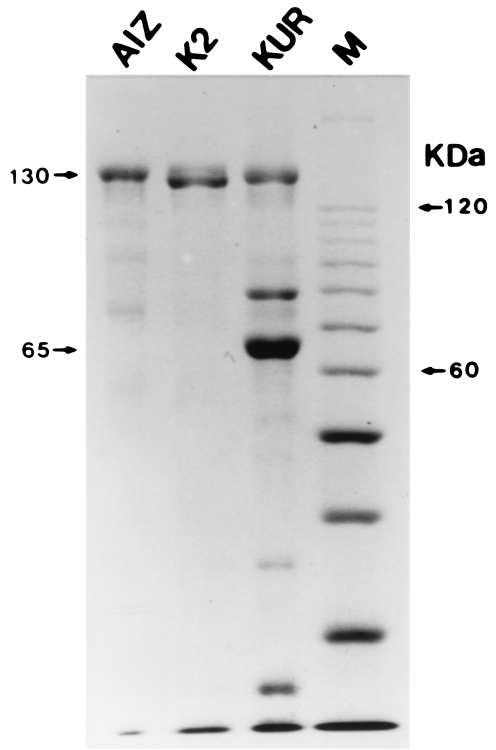

Fig. 2. SDS-PAGE analysis of parasporal inclusions of *B. thuringiensis* strains. Lanes: AIZ, *B. thuringiensis* ssp. *aizawai*; K2, K2; KUR, ssp. *kurstaki* HD-1. M indicates 10 kDa protein ladder (Gibco BRL).

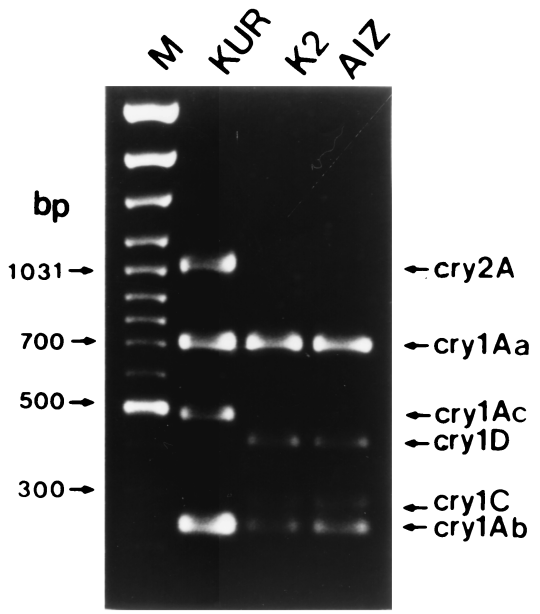

Fig. 3. Detection of *cry* genes of *B. thuringiensis* K2 by PCR. Lanes: KUR, *B. thuringiensis* ssp. *kurstaki* HD-1; K2, K2; AIZ, ssp. *aizawai*. M indicates 100 bp DNA ladder (Promega Co.).

Table 1. Toxicity of *B. thuringiensis* K2 against *Spodoptera exigua* and *Culex pipiens*

	LC_{50} ($\times 10^5$ cfu/ml)			
Insect	K2	KUR[a]	AIZ[b]	ISR[c]
S. exigua	10.7	185.3	15.1	ND
C. pipiens	3.2	9300	ND	2.7

[a] KUR, *B. thuringiensis* ssp. *kurstaki* HD-1.
[b] AIZ, *B. thuringiensis* ssp. *aizawai*.
[c] ISR, *B. thuringiensis* ssp. *israelensis*.

production and have been determined to be Tra^+ [1, 23]. And self-transmissible plasmids are also able to mobilize nonconjugative plasmids [23]. Therefore, these transmission of genes or/and plasmids could well have promoted recombination events leading to the present hybrid gene structure of K2.

In the bioassay to *P. xylostella*, all *B. thuringiensis* strains, K2, ssp. *kurstaki* HD-1, and ssp. *aizawai*, showed 100% mortality at the concentration of 1×10^6 cfu/ml. In the bioassay against *S. exigna*, K2 spore-crystal complex showed the high toxicity with an estimated LC_{50} of 10.7×10^5 cfu/ml (Table 1). The isolate K2 was significantly more toxic than ssp. *kurstaki* HD-1 and similar with ssp. *aizawai*. From the PCR results, the toxicity of *B. thuringiensis* K2 against *S. exigua* was due to *cry1C* and *cry1D* gene expression.

Because high mosquitocidal activity of K2 was checked at a primary screening, quantitative bioassay on *C. pipiens* was performed. *B. thuringiensis* ssp. *kurstaki* HD-1 and ssp. *israelensis* were used for controls. Interestingly, K2 was a strain of *B. thuringiensis* ssp. *kurstaki*, but had similar toxicity with ssp. *israelensis*. *B. thuringiensis* ssp. *israelensis* inclusions are toxic to mosquito and blackfly larvae. The crystals of *B. thuringiensis* ssp. *israelensis* are composed of four major polypeptides referred to as Cry4A, 4B, 10A, 11A, and CytA [9, 16]. But no mosquitocidal crystal protein gene was detected in PCR analysis of the isolate K2. One explanation for high mosquitocidal activity of K2 was the activity and cytolytic effect of Cry1C protein. Cry1C from *B. thuringiensis* ssp. *aizawai* HD-229 was toxic to some *Aedes* and *Culex* species and solubilized and proteolytically activated Cry1C was also cytolytic to *Aedes*- and *Anopheles*-derived cell lines [20]. Another possible explanation concerned the secretion of β-exotoxin or thuringiensin. The toxicity of this compound is thought to be due to inhibition of DNA-directed RNA polymerase by competition with ATP [11]. In mosquitoes, the β-exotoxin acts as a larvicide and adulticide when digested. Because the production of β-exotoxin is a strain-specific property rather than a serotype-specific property [21], additional research with β-exotoxin activity of K2 should be nec-

essary. We are now looking for the crystal gene structure of K2, specially for *kurstaki*-originated *cry1C*- and *cry1D*-type genes.

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