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Molecular Characterization of A Novel *Bacillus thuringiensis* Strain from China

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A strain of *Bacillus thuringiensis* that showed significantly high toxicity to *Plutella xylostella* was isolated from a dust sample collected from Chinese tobacco warehouse and characterized. The isolate named *B. thuringiensis* LY-99 was determined to belong to subsp. *alesti* (H3a3c) by an H antisera agglutination test and produced bipyramidal inclusions. Plasmid and crystal protein patterns of the LY-99 were different from those of the reference strain, subsp. *alesti*. PCR analysis with specific primers revealed that this isolate contained abundant *cry* genes including *cry1Aa*, *cry1Ac*, *cry1B*, *cry1D*, *cry1E*, *cry1F* and *cry2* genes, which was absolutely different from *cry* gene profile of the subsp. *alesti*. In addition, insecticidal activity of the LY-99 against *P. xylostella* larvae was about 44 times higher than that of the subsp. *alesti*.

Key words: *Bacillus thuringiensis* LY-99, *Bacillus thuringiensis* subsp. *alesti*, *cry* gene, *Plutella xylostella*, Insecticidal activity

Introduction

Bacillus thuringiensis, a gram-positive spore-forming soil bacterium, is an entomopathogen and has been used as an insecticidal agent for decades in commercial agriculture, forest management, and mosquito control (Schnepf *et al.*, 1998). Its insecticidal activity is primarily due to the insecticidal crystal proteins (ICPs) formed during the stationary and sporulation phase of the growth cycle (Hofte and Whiteley, 1989). The ICPs include the more prevalent Cry (crystal) proteins and the Cyt (cytolytic) proteins, which are encoded by *cry* and *cyt* genes, respectively. Although proteins in the Cry and Cyt families have entirely different structures (Li *et al.*, 1991, 1996), modes of action of both families are thought to be similar (Knowles, 1994).

More than 3,000 insect species within 16 orders were demonstrated to be susceptible to different *B. thuringiensis* ICPs (Zhipeng *et al.*, 2004). However, the development of pest resistance has threatened the effectiveness of *B. thuringiensis* toxins used for insect pest control. Although field resistance has been found in a small number of insects (Ferré *et al.*, 1991; Herron *et al.*, 2001), there was evidence of insect resistance to Cry proteins in laboratory conditions (Tabashnik *et al.*, 2000). The problem with insect resistance has become even more apparent in relation to the expression of *cry* genes in transgenic plants (Jouanin *et al.*, 1998; Leroy *et al.*, 2000; Groot and Dicke, 2002). Therefore, the isolation and description of new isolates of *B. thuringiensis* with toxic properties that different from those already used as bioinsecticides is particularly important from an ecological as well as an economical point of view.

So far, more than 60,000 *B. thuringiensis* strains have been isolated in worldwide, and 312 *cry* genes and 22 *cyt* genes have been reported and classified into *cry1* to *cry47* and *cyt1* to *cyt2* according to the degree of amino acid sequence similarity. Also, on the basis of their flagellar (H) antigens, *B. thuringiensis* has been classified in 69 serotypes comprising 82 serovars (Lecadet *et al.*, 1999).

In this study, a novel *B. thuringiensis* isolate, LY-99,

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which is highly toxic to *Plutella xylostella*, an important lepidopteran pest of vegetables worldwide, was isolated and characterized.

Materials and Methods

Bacterial strains and growth media

B. thuringiensis isolate named LY-99 was selected from a dust sample from tobacco warehouse in China according to its toxicity against lepidopteran insects. A reference strain, *B. thuringiensis* subsp. *alesti* (H3a3c), was kindly provided by Dr. Ohba (Institute of Biological Control, Faculty of Agriculture, Kyushu University, Japan). For preparation of parasporal inclusions and purification of plasmid DNA, GYS and SPY media were used, respectively (Chang *et al.*, 1998).

Preparation of H antisera and H agglutination

H antisera of previously reported strains of 33 serotypes (H1 to H27) of *B. thuringiensis* were prepared as described by Ohba and Aizawa (1978). H antisera-antigen agglutination was performed using 96-well plates (Chang *et al.*, 1998; Li *et al.*, 2002).

Plasmid DNA extraction

The plasmid DNA of *B. thuringiensis* strains was isolated using QIAGEN midi prep. Kit (QIAGEN Co., Germany) according to the manufacturer's protocol. The total plasmid DNA patterns of *B. thuringiensis* strains were analyzed on 0.8% agarose gel.

Morphological observation and protein analysis

Parasporal inclusions were purified by a slightly modified method of Thomas and Ellar (1983) using a discontinuous 60 to 85% sucrose gradient. Crystal morphology was examined by phase-contrast microscopy and scanning electron microscopy. For SDS-PAGE samples, cells were cultured in GYS medium at 30°C and harvested after autolysis. SDS-PAGE was performed on a 10% polyacrylamide gel as described by Laemmli (1970). The gel was stained with 0.1% Coomassie brilliant blue (Sigma Co., USA).

PCR analysis

The 21 *cry*-type gene specific primer sets used in the PCR analysis for the identification of the specific δ-endotoxin genes (*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B*, *cry1C*, *cry1D*, *cry1E*, *cry1F*, *cry1G*, *cry2*, *cry3A*, *cry3B*, *cry3C*, *cry3D*, *cry4A*, *cry4B*, *cry4C*, *cry4D*, *cry5*, *cyt1* and *cyt2*) were synthesized as previously reported (Carozzi *et al.*, 1991; Gleave *et al.*, 1993; Kalman *et al.*, 1993; Ceron *et al.*, 1994, 1995). Another two oligonucleotide primers, cry1Ae-F (5'-TCGAATTGAATTTGTTCC-3') and cry1Ae-R (5'-CGGAATAATTGCTTCCATAAG-3'), were designed based on the conserved sequences of the known *cry1Ae* gene. The PCR reaction was carried out with 250 ng of plasmid DNA, 100 pM of each primer and PCR PreMix kit (Bioneer Co., Korea) in a 20 μl PCR mixture for 30 thermal cycles (94°C for 1 min, 45 to 56°C for 1 min and 72°C for 3 min). Amplification was accomplished with the DNA Thermal Cycler (Perkin Elmer Cetus, USA).

Insect bioassays

Primary bioassay and quantitative bioassay were performed as previously described (Chang *et al.*, 1998; Li *et al.*, 2002). In primary bioassay, *Spodoptera exigua* and *P. xylostella* were tested. In a quantitative bioassay, sporulated cultures of *B. thuringiensis* were serially diluted in 0.01% Triton X-100 (vol/vol), and 200 μl aliquots of serial dilutions were applied to the surface of artificial diets for 30 larvae each of third instar *P. xylostella* and mortality was recorded after 48 hrs. All tests were performed with spore-parasporal inclusion suspensions and independently repeated three times. Statistical analysis of data was performed with probit analysis (Russell *et al.*, 1977).

Results and Discussion

Up to date, several ten thousands of *B. thuringiensis* strains have been isolated from all kinds of environments in the world. The isolation of *B. thuringiensis* strain from warehouse had been reported previously (Meadows *et al.*, 1992; Hongyu *et al.*, 2000). A strain of *B. thuringiensis*, named LY-99, isolated from a dust sample from tobacco warehouse of China, was selected on the basis of its insecticidal activity against *P. xylostella*. Serological study indicated that H antigenic structure of the isolate LY-99 was identical to that of *B. thuringiensis* subsp. *alesti* (H3a3c). The crystal morphology of the LY-99 analyzed by phase-contrast microscopy and scanning electron microscopy was found to be typical bipyramidal, which similar to that of the reference strain, subsp. *alesti* (data not shown).

However, the plasmid pattern of LY-99 was different from that of the reference strain, subsp. *alesti* (Fig. 1A). In addition, SDS-PAGE analysis of parasporal inclusion proteins of LY-99 revealed that the crystal protein profile of the LY-99 was different from that of subsp. *alesti* (Fig. 1B). Although both of the LY-99 and subsp. *alesti* showed a major band of about 130 kDa in size, the isolate LY-99 had another weak band of about 65 kDa in size, which

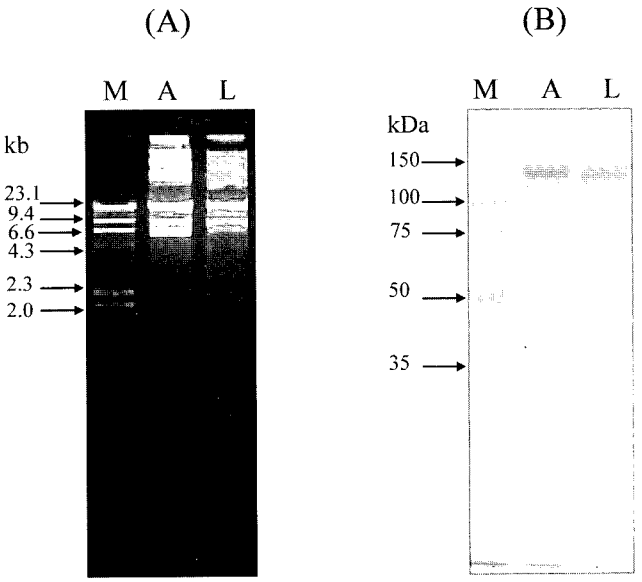

Fig. 1. Plasmid DNA pattern (A) and SDS-PAGE analysis of parasporal inclusions (B) of *B. thuringiensis* isolate LY-99. Lanes: M, Lambda DNA digested with *Hin*dIII (A) and protein molecular weight mark (B); A, *B. thuringiensis* subsp. *alesti*; L, *Bacillus thuringiensis* LY-99.

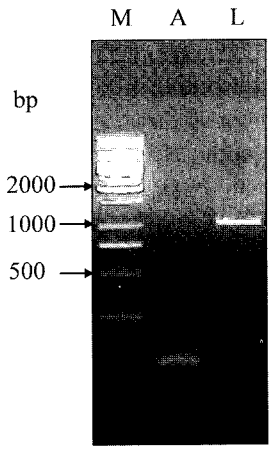

Fig. 3. PCR amplification of *B. thuringiensis* subsp. *alesti* and LY-99 with *cry2* gene specific primer set. Lanes: M, 1 kb DNA ladder; A, *B. thuringiensis* subsp. *alesti*; L, *Bacillus thuringiensis* LY-99.

was not found in subsp. *alesti*. These results strongly suggested that the isolate LY-99 could contain somewhat different crystal protein profile from the reference strain, subsp. *alesti*.

To identify crystal protein genes of the LY-99, PCR analysis was performed with *cry* gene-specific primer sets. While the reference strain, subsp. *alesti*, only showed the product of *cry1Ae* gene (Fig. 2A) as reported previously (Lee and Aronson, 1991), the products of *cry1Aa, cry1Ac, cry1B, cry1D, cry1E, cry1F* (Fig. 2B) and *cry2* genes (Fig. 3) were amplified from the LY-99 as the expected size. The two bands of about 130 kDa and 65 kDa in SDS-PAGE would correspond to Cry1- and Cry2-type proteins, respectively. Although the LY-99 has the same serotype with subsp. *alesti*, no the PCR product of *cry1Ae* gene was found with specific primer (Fig. 2B).

The insecticidal activities of the LY-99 and subsp. *alesti* were tested against *P. xylostella* (Table 1). LC_{50} value of the isolate LY-99 against *P. xylostella* larvae was about 44 times higher than that of subsp. *alesti*. The specific crystal genes working against *P. xylostella* are *cry1Aa, cry1Ab, cry1Ac, cry1B*, and *cry1C* (Entwistle *et al.*, 1993). As seen in PCR analysis, *cry1Aa, cry1Ac, cry1B, cry1D, cry1E, cry1F*, and *cry2* genes were included by LY-99, resulting high toxicity to *P. xylostella*. *B. thuringiensis* biopesticide

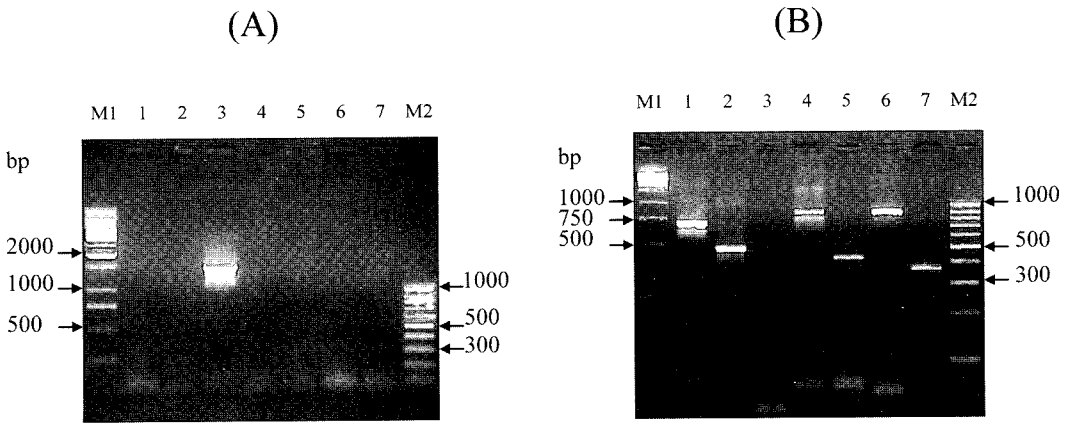

Fig. 2. Detection of *cry1*-type gene content of *B. thuringiensis* subsp. *alesti* (A) and LY-99 (B) by PCR using specific primer sets. Lanes: M1, 1 kb DNA ladder; 1, *cry1Aa* specific primers; 2, *cry1Ac* specific primers; 3, *cry1Ae* specific primers; 4, *cry1B* specific primers; 5, *cry1D* specific primers; 6, *cry1E* specific primers; 7, *cry1F* specific primers; M2, 100 bp DNA ladder.

Table 1. Median lethal concentration (LC_{50}) of *B. thuringiensis* LY-99 against third instar larvae of *P. xylostella*

Strain	LC_{50} ($\times 10^4$ CFU/ml)	95% Fiducial limits ($\times 10^4$ CFU/ml)
B. thuringiensis subsp. *alesti*	75.4	31.3-167.7
B. thuringiensis LY-99	1.7	0.4-6.6

products composed of multiple endotoxins that interact with distinct membrane receptors may be less likely to lead to resistant insect populations. Therefore, multiple *cry* gene content of the LY-99 could not only improve the insecticidal activity against *P. xylostella* but also postpone the resistance of insect pest to *B. thuringiensis*. For this reasons, this isolate could be a potential strain used for the industrial production to control one of the most important cosmopolitan pest, *P. xylostella*.

The existence of seven kinds of *cry* genes (*cry1Aa, cry1Ac, cry1B, cry1D, cry1E, cry1F,* and *cry2* genes) in *B. thuringiensis* isolate belonging to subsp. *alesti* was reported for the first time in this study. To further confirm these *cry* genes, and look for novel *cry* genes, the cloning and sequencing of all existing *cry* genes from LY-99 should be proceeded.

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