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Novel *Bacillus thuringiensis* serovar *aizawai* strains isolated from mulberry leaves in Indonesia

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Abstract
Bacfllus thuringiensis isolates have been recovered from numerous sources, including soil, grain dust, plant leaves, diseased insect larvae from insectaries, and Sericulture environments. During a study of *B. thuringfensis* isolated from mulberry leaves from Indonesia, we found two serovar aizawni isolates. One of the serovar *aizawaf* isolates (Bun 1-14), which was a crystal consisting mainly of 69 kDa peptides, exhibited mosquitocidal activity, while another isolate (Bun 2-1) did not. Both isolates were analyzed by PCR. Although these isolates produced proteinaceous crystals. no *cry* genes, known as *cryI, cryII, cryIII* and *cryIV,* were detected. It appears these strains contain novel *cry* genes that are responsible for the unique insecticidal activity.

Key words: *Bacillus thuringiensis,* serovar *aizawai,* mulberry leaf, mosquitocidal

INTRODUCTION

Bacillus thuringiensis spores and/or inclusion bodies typically express insecticidal activity, and several strains are used on a global basis to control larval forms of major agricultural insect pests. In recent years, the need for environmentally safe pesticides has encouraged the search for new strains of *B. thuringiensis* with different target spectra, These novel isolates will augment the current commercial *B. thuringiensis* strains that exhibit various degrees of activity toward larvae of the insect orders of Lepidoptera, Diptera, and Coleoptera.

More than 1,000 isolates of *B. thuringiensis* have been categorized into 2 major groups (Filosa and Dengler, 1972; Saga and Yanagisawa, 1982). These isolates have been recovered from numerous sources, including soils, grain dust, diseased insect larvae from insectaries and sericulture environments. The principal source of novel *B. thuringiensis* isolates has been soil. Hastowo et al. (1992) reported that 135 strains of *B. thuringiensis* were isolated from soils of sericultural and natural environments in various regions in Indonesia. Smith and Couche (1991) isolated *B. thuringiensis* from the phylloplane of deciduous and conifer trees, as well as of other plants.

During a study of *B. thuringiensis* isolated from mulberry leaves in Indonesia, a novel isolate was found which exhibited high mosquitocidal activity. In this paper, a comparative study of *B. thuringfensis* serovar *alzawai* strains is described.

MATERIALS AND METHODS

Bacteria and culture media. The reference strains of *B. thuringiensis* serovar *aizawai* IPL and serovar *israelensis* ONR-60A (lizuka et a1., 1982) were maintained in this laboratory. The isolates of *B. thuringiensis,* serovar *aizawai,* were identified by H-serotype. II-antisera to the reference strain of *B. thuringiensis* serovar *aizawai* IPL was prepared according to the method of Ohba and Aizawa (1978). For H-serotyping of the strains, actively motile bacteria were selected by passing through craigie's tubes at 37°C for 24 h. Slide agglutination was performed by mixing one drop of 3 to 4 h-old flagellated broth culture of the bacteria on a glass slide with one drop of 20 to 50-fold dilution of H antiserum. Specific H agglutination was determined 3 to 5 min after mixing.

The isolates were obtained from mulberry leaves collected in West Java, Indonesia, using

the leaf-lift technique (Smith and Couche, 1991). Leaves were trimmed to fit inside a 100 mm petri dish. Abaxial leaf surfaces were placed in contact with nutrient agar, and a sterile, perforated stainless steel disk was placed on the leaf sections to ensure maximum contact with the agar. The lid was replaced, and samples were incubated at 30°C overnight. To prepare the sporulated culture, bacteria were grown on nutrient agar, pH 7.0, at 30°C for 4 days. Formation of spores and parasporal inclusions were monitored with a phase-contrast microscope.

Parasporal inclusion morphology and biological activity. Isolates were examined with a HITACHI S-800 scanning electron microscope at a magnification of 10,000 X , according to the method presented by lizuka et a1. (1982).

The eggs of *Anopheles stephensi* (strain BEECH) were kindly supplied by Dr. H. Saitoh (Fukuoka Industrial Technology Center, Japan). A toxicity test with the mosquito, *A. stephensi,* was done by introducing ten 2nd-stadium larvae into a test tube containing 10 ml bacterial suspension. The bacterial suspension were prepared by the following procedure: the strains were cultured on a nutrient agar plate and sporulated cultures were harvested by centrifugation at 10,000 x Q for 10 min at 4°C. The pellets were washed three times by centrifugation in 1 M NaC1 at 4°C, then dried at room temperature for 7 days. The dried pellets were weighed and suspended in distilled water with 2-fold serial dilutions. Larvae were kept unfed, and the mortalities were scored after 24 h of incubation at 22°C. Assays were done in triplicate and the 50% lethal concentration values (LC_{50}s) were determined by probit analysis (Finney, 1971).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The purity of parasporal inclusion using Percoll (Pharmasia) (Baba et al., 1990) was monitored with a phase-contrast microscope. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of parasporal inclusion proteins was performed by the method developed by Laemmli (1970), using a 10a running gel with a 3% stacking gel. After electrophoresis, the gel was stained with 0.04% Coomassie brilliant blue (Sigma Chem. Co.). The following prestained reference proteins (Bio-rad Laboratories) were used as molecular markers: myosin (200 kDa), Q-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa).

Polymerase chain reaction (PCR) procedures. The PCR technique was used to identify the contents of *B. thuringiensis* serovar *aizawai* Bun 2-1 and Bun 1-14, using oligonucleotide primers specific to *cryl* (Kalman et al., 1993), *cryII* (Asano et al., 1993) and *cryIV(Asano,* 1996). Identificatlon of *cry* genes in a genomic DNA sample extracted from *B. thuringiensis* strains was based on unique-size DNA fragments amplified by PCR for each *cry* gene (Asano, 1996).

RESULTS AND DISCUSSION

H-serotyping

The isolates of Bun 2-1 and Bun 1-14 gave a positive reaction in the H7 serum agglutination test and are apparently *B. thuringiensis* serovar *aizawai* types. Scanning electron microscopy (SEM) revealed that *B. thuringiensis* serovar *aizawai* Bun 2-1 and serovar *aizawaf* Bun 1-14 produced irregular-shaped crystal proteins (Fig. 1).

Quantitative **toxicity** test of isolates

The serovar *aizawai* strains and serovar *fsraelensis* ONR-60A were bioassayed with 2nd-stadium larvae of *Anopheles stephensi*. To remove the Q-exotoxin activity, sporulated cultures were washed three times with 1 M NaC1 and dried. Serovar *aizawai* Bun 1-14 showed toxic activity against *A. stephensi,* while serovar *aizawai* IPL and *aizawai* Bun 2-1 were not toxic to this insect. The LC5 values for *B. thuringien-Sfs* serovar *israelensis* ONR-60A were similar to

Table 1. Toxicities of crystal proteins from four strains of *B. thuringiensis* to 2nd-stadium larvae of *Anopheles stephensi*

Serovar	$LC_{,,}$ Jg/ml)‘	Slope
israelensis ONR-60A	0.027 (0.014—0.060)	2.588*0.849
aizawai IPL	5[b]	
aizawai BUN1-14	0.057 (0.035—0.088)	3.507*1.032
aizawai BUN2-1	5‘	

Values in parentheses are fidual limits 950a.
Insufficient mortality to calculate LCMC.

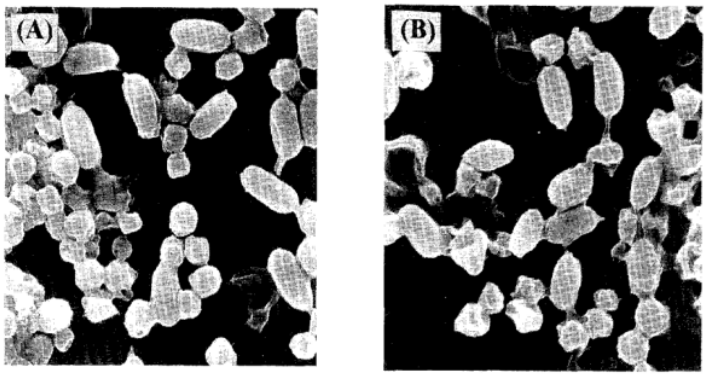

Fig. 1. Scanning electron microscopy showing spores and parasporal crystals of *B. thuringiensis* serovar *aizawai* Bun 2-1 (A), and serovar *aizawai* Bun 1-14 (B). Bar; *3* pm.

those for serovar *aizawai* Bun 1-14 (Table 1). Ohba (1996) reported that the serovar *aizawai* strains which were isolated from mulberry leaves had a moderate toxicity against mosquito larvae. Serovar *aizawai* Bun 1-14 was a novel strains which was isolated from mulberry leaves.

SDS-PAGE analysis

SDS-PAGE analysis of parasporal inclusion purified from serovar *aizawai* Bun 1-14 revealed significant differences in protein composition, compared with serovar *aiznwai* Bun 2-1. The crystal of serovar *aizawai* Bun 1-14consisted mainly of 69 kDa peptides, while serovar *aizawai* Bun 2-1 consisted mainly of 130—65 kDa peptides (Fig. 2). However, there was no overlap in the profiles of the other crystal proteins with those of these two *B. thuringiensis* serovar *aizawai* strains.

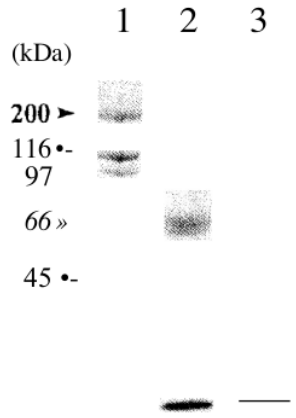

Fig. 2. SDS-PAGE of crystal proteins of *B. thuringiensis*. Lane 1, molecular weight marker (in kilodaltons); lane 2, *B. thuringiensis* serovar *aizawai* Bun 2-1; lane 3, serovar *aizawaf* Bun 1-14.

Identification of *cry* genes in *B. thuringiensis* serovar *aizawai* strains

In the identification of *cry* genes using PCR, serovar *nizawni* Bun 1-14 and Bun 2-1 did not amplify *cryIC*, *cryIIA* and *cryIV* genes. However, serovar *aizawai* Bun 1-14 had a high toxicity against *A. stephensi*. In future studies, new *cry* genes will be cloned from Bun 1-14 and analyzed for toxicity of insecticidal proteins.

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