Appl. Entomol. Zool. 33 (1): 111-114 (1998)

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

Akhmad Rizali, Shin-ichiro Asano, Ken Sahara, Hisanori Bando, Bibiana W. Lay,[1] Sugyo Hastowo[1] and Toshihiko Iizuka

Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
[1]Bogor Agricultural University, Bogor 16151, Indonesia

(Received 2 June 1997; Accepted 29 September 1997)

Abstract

Bacillus 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. thuringiensis* isolated from mulberry leaves from Indonesia, we found two serovar *aizawai* isolates. One of the serovar *aizawai* 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. thuringiensis* serovar *aizawai* strains is described.

MATERIALS AND METHODS

Bacteria and culture media. The reference strains of *B. thuringiensis* serovar *aizawai* IPL and serovar *israelensis* ONR-60A (Iizuka et al., 1982) were maintained in this laboratory. The isolates of *B. thuringiensis*, serovar *aizawai*, were identified by H-serotype. H-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×, according to the method presented by Iizuka et al. (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×*g* for 10 min at 4°C. The pellets were washed three times by centrifugation in 1 M NaCl 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 10% 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), β-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 *cryI* (Kalman et al., 1993), *cryII* (Asano et al., 1993) and *cryIV* (Asano, 1996). Identification 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 *aizawai* Bun 1-14 produced irregular-shaped crystal proteins (Fig. 1).

Quantitative toxicity test of isolates

The serovar *aizawai* strains and serovar *israelensis* ONR-60A were bioassayed with 2nd-stadium larvae of *Anopheles stephensi*. To remove the β-exotoxin activity, sporulated cultures were washed three times with 1 M NaCl 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 LC_{50} values for *B. thuringiensis* 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_{50} (μg/ml)[a]	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[b]	

[a] Values in parentheses are fidual limits 95%.
[b] Insufficient mortality to calculate LC_{50}.