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Synergistic effect of *Xenorhabdus nematophila* K1 and *Bacillus thuringiensis* subsp. *aizawai* against *Spodoptera exigua* (Lepidoptera: Noctuidae)

Sungchae Jung, Yonggyun Kim *

Department of Bioresource Sciences, Andong National University, Andong 760-749, Korea

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Abstract

Xenorhabdus nematophila K1 (*Xn*) is the symbiotic bacterium of an entomopathogenic nematode, *Steinernema carpocapsae*, and causes fatal septicemia of the target insect. Our study showed that when orally ingested, the symbiotic bacterium could exhibit insecticidal toxicity against young larvae of *Spodoptera exigua*. The oral toxicity of *Xn* alone was analyzed in terms of its antibiotic activity to the target insect's intestinal bacterial flora because *Xn* alone did not penetrate into the hemocoel of the insect. An intestinal bacterium was isolated from *S. exigua* and identified as *Bacillus cereus* K1. Owing to the symbiotic role of *B. cereus* for development and survival of young *S. exigua* larvae, the inhibitory activity of the orally ingested *Xn* against *B. cereus* can be suggested as a pathogenic pathway. *Bacillus thuringiensis* subsp. *aizawai* (*Bta*) effectively killed the third instar larvae of *S. exigua*, but did not cause high mortality of the fifth instar larvae. A mixture of *Xn* and *Bta* fed to the fifth instar larvae resulted in significant synergistic effect. The synergistic effect of the *Xn* and *Bta* mixture could be explained by the passage of *Xn* into the hemocoel through the damaged midgut cells caused by the *Bta* infection. Our study suggests a novel insecticidal activity of *Xn* in the gut as well as being a hemocoelic pathogenic agent. It also demonstrates a synergism between two entomopathogenic bacteria whereby *Bta* assists in allowing *Xn* to penetrate into insect's hemocoel.

Keywords: *Bacillus cereus*; *Bacillus thuringiensis*; Septicemia; Beet armyworm; Entomopathogenic bacteria; Entomopathogenic nematode

1. Introduction

Xenorhabdus nematophila (Poinar and Thomas) (*Xn*) is a Gram-negative bacterium possessing potent entomopathogenic activity (Thomas and Poinar, 1979; Akhurst, 1980, 1983). Its natural nematode host, *Steinernema carpocapsae* (Weiser), delivers the bacterium into the target insect's hemocoel (Bird and Akhurst, 1983; Boemare et al., 1997). In the hemocoel, the bacterium multiplies and causes septicemia, which ultimately kills the infected insect (Dunphy and Webster, 1988; Park and Kim, 2000). To be effective, the nematode host has been regarded as an indispensable agent for *Xn* to be delivered into target insect's hemocoel.

Bacillus thuringiensis Berliner is a Gram-positive, endospore-forming bacterium containing δ-endotoxin that targets the insect midgut epithelium (Jenkins and Dean, 2000). Insects infected with *B. thuringiensis* show cell lysis of the midgut epithelial tissues and exhibit characteristic fatal signs and symptoms such as gut paralysis, cessation of feeding, and septicemia (Bravo et al., 2005).

The beet armyworm, *Spodoptera exigua* (Hübner), is a lepidopteran pest causing serious damage on major vegetable crops and is not effectively controlled by chemical pesticides because of its wide range of resistance against most commercial insecticides (Brewer and Trumble, 1989; Van Laecke and Degheele, 1991). Moreover, the late larval instars are significantly less susceptible to the chemical insecticides (Kim et al., 1998). *B. thuringiensis* endotoxins vary in their toxicity during larval development of lepidopteran species (Gilliland et al., 2002). For example,

* Corresponding author. Fax: +82 54 823 1628.
E-mail address: hosanna@andong.ac.kr (Y. Kim).

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B. thuringiensis subsp. *kurstaki* Cry1A(c)-type δ-endotoxin, which is highly toxic to many lepidopteran pests, was not very efficacious against *S. exigua* except in its early larval stages (Lastra et al., 1995).

To effectively control *S. exigua* especially in the late in stars with biological control agents, our research focused on a mixture treatment of *Xn* and *B. thuringiensis* subsp. *aizawai (Bta)*. When orally fed, *Bta* may play a role in assisting *Xn* to enter from gut lumen to the hemocoel of the insects by the breakdown of the midgut epithelium. Then both pathogenic actions of *Bta* and *Xn* can be expressed, which may result in significant synergism between the two bacterial species. Our research showed a highly synergistic effect of these two biological agents and explained how the synergistic effects worked in the infected *S. exigua*. In addition, our research suggests a novel approach to use *Xn* to control insect pests without help of its nematode host.

2. Materials and methods

2.1. Insect and bacterial culture

The beet armyworm, *S. exigua*, used in this study originated from a field population infesting welsh onion in Andong, Korea. The larvae were reared on artificial diet (Gho et al., 1990) at 25 °C and the adults were fed 10% sucrose solution.

X. nematophila K1 (*Xn*) was isolated from its nematode host, *S. carpocapsae* and characterized (Park et al., 1999; Ji et al., 2004a). The primary form of the bacterium was cultured on tryptic soy agar (TSA) (Difco, Detroit, MI, USA) and freeze-dried for later use. For formulation of *Xn*, the bacterium was cultured in tryptic soy broth for 48 h at 25 °C until their stationary phase and freeze-dried using Vacuum Tray Freeze Dryer (Bondiro 1SE, Ilsin Co., Busan, Korea) for three days. The bacterial colonies from the freeze-dried material were cultured on TSA for 24 h at 25 °C and the number of colonies formed using a dilution series was expressed as colony-forming units (cfu) per the dried formulation weight (μg). For bioassay, the weighed bacterial formulation was resuspended in sterile water and the resulting suspension was prepared as μg/ml through serial dilutions.

Bta GB413 (GreenBioTech, Chungju, Korea) was donated and used in our tests. Its active ingredient was expressed as cfu/μg, even though it consisted of the bacterial spore and endotoxin.

2.2. Bioassay for bacterial pathogenicity

All pathogenic bioassays used the dipping method, in which the artificial diet was cut into small pieces (about 1 cm^3) and soaked into the predetermined concentrations of bacterial suspension for 5 min. After drying in the dark condition for about 10 min, the treated diet was given to the test larvae. Each bacterial concentration was fed to 10 larvae per replication and run independently three times. Mortality was determined every 24 h after treatment. Larvae were considered dead or dying if they did not move in a coordinated manner when prodded with a blunt probe. Results were analyzed using Probit analysis (Raymond, 1985).

2.3. Identification of a gut bacterial isolate with biochemical characters

Gut contents of the three fifth instar larvae of *S. exigua* were extracted and filtered on sterile cheesecloth. The filtrate was diluted with sterile water and plated on TSA, which was incubated in an anaerobic incubator (AALC, Cay Lab Products Inc., Grass Lake, MI, USA) at 25 °C for two days.

Gram stain of the bacterial isolate was performed by the method of Bensen (1990). The activities of catalase and oxidase were examined by the procedure of Schaad (1988). To identify the species, acid production characters from different carbon sources were further analyzed by the colorimetric method using GP microplate (Biolog, Hayward, CA, USA) and compared with the characters of *Bacillus cereus* Frankland and Frankland (a highly matched species in this study) described in Bergey's manual (Krieg and Hort, 1984). Fatty acid compositions of the bacterial cell wall were analyzed by Sherlock system (HP 6890 series, MIDI, Inc., Newark, DE, USA), where the preparation steps followed the method described in the instrument manual.

2.4. Bacterial identification by 16S rDNA sequence

Total genomic DNA was extracted from the bacterial isolate (Sambrook et al., 1989). The bacterial cells were lysed and then digested with proteinase K (50 μg/ml). After phenol extraction, the genomic DNA was precipitated with ethanol and dried under vacuum. The purified DNA was resuspended with deionized water and used for PCR template. Universal PCR primers (Weisburg et al., 1991) were used to amplify the 16S rDNA of the bacterial isolate. Forward and reverse primers were 5′-GAA GAG TTA GAT CTT GGC TC-3′ and 5′-AAG GAG GTG ATC CAG CCG CA-3′, respectively. All PCR tubes were kept on ice, and the following PCR mixture was added to each tube: 5 μl of 10 × PCR buffer, 4 μl of dNTP mixture (2.5 mM), 2 μl of 25 pmol forward primer, 2 μl of 25 pmol reverse primer, 1 μl of *Taq* polymerase (5 units/μl), 2 μl of template DNA, and 34 μl of distilled water. Mineral oil (20 μl) was placed on top of the solution in the tube to minimize evaporation. PCR (PTC-100 MJ Research, Watertown, MA, USA) conditions were 35 cycles of 94 °C for 1 min, 68 °C for 1 min, and 72 °C for 1 min. The PCR product was cloned into a TA vector (pGEM®, Promega, Madison, WI, USA). Sp6 and T7 sequencing primers were used for bidirectional sequencings. All sequencings were performed by a DNA sequencing company (Macrogen, Daejon, Korea). The