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Biodiversity and pathogenicity of bacteria associated with the gut microbiota of beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae)

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ABSTRACT

In order to find an effective and environmentally friendly biocontrol agent against *Spodoptera exigua*, we isolated and identified a total of 15 different bacterial species belonging to phyla *Firmicutes*, *Proteobacteria* and *Bacteroidetes*. According to the phenotypic, genotypic and phylogenetic properties, bacterial isolates were identified as *Bacillus cereus* (Se1), *Lysinibacillus macroides* (Se2), *Pseudomonas geniculata* (Se3), *Paenibacillus tylopili* (Se4), *Staphylococcus succinus* (Se5), *Acinetobacter soli* (Se6), *Chryseobacterium indologenes* (Se7), *Bacillus toyonensis* (Se8), *Serratia marcescens* (Se9), *Paenibacillus amylolyticus* (Se10), *Paenibacillus xylanexedens* (Se11), *Enterobacter ludwigii* (Se12), *Bacillus thuringiensis* (Se13), *Bacillus thuringiensis* (Se14) and *Lysinibacillus fusiformis* (Se15). Screening of bacterial isolates for insecticidal potential was conducted at 10^9 cfu ml^{-1} bacterial concentration. The highest larvacidal effect was obtained with *Bacillus thuringiensis* Se13 with 100% mortality. In the dose response experiments performed with this bacterium, the median lethal concentration (LC_{50}) was estimated as 7.5×10^4 cfu ml^{-1} against 3rd instar larvae of the pest at 10 days post treatment. The median lethal time (LT_{50}) value of 10^9 cfu ml^{-1} bacterial concentration was also determined as 1.59 days. Phase-contrast and scanning electron microscope studies exhibited that *B. thuringiensis* Se13 produced different shape and size crystals (bipyramidal, cubic and spherical). Phylogenetic analysis of *cry*1 and *cry*2 gene content of this isolate displayed that *B. thuringiensis* Se13 had 99% homology with *cry*1Ac and *cry*2Aa, respectively. Finding from this study indicated that *B. thuringiensis* Se13 appears to be a promising microbial control agent for use against *S. exigua*.

1. Introduction

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) is one of the best known polyphagous agricultural pest. Larvae of the pest feed on the foliage and fruits of plants causing economically important yield losses in cultivated crops such as cotton, sunflower, maize, pigweed, sugar and table beet in many countries [1,2]. *S. exigua* larvae feed in terminal clusters of host crops and often do considerable damage before they are noticed. Leaves may be skeletonized and almost completely consumed.

Many control strategies have been used to manage the beet armyworm, with chemical insecticides being the most commonly used tactic. Due to its polyphagous nature, this pest has been exposed to many insecticides for years. Interestingly, beet armyworm has developed resistance to many of these, including chlorinated hydrocarbons, organophosphates, spinosads, pyrethroids, and benzoylphenylureas [3,4]. In addition, chemical insecticides are not the safest alternative, since they cause damage to the environment and they are harmful to non-target organisms including human. Along with chemical control, several biological insecticides have been evaluated to control beet armyworm. Pheromones have been used in management strategies for some insect pests, whether used for mating disruption or monitoring of adult flight. The beet armyworm and its pheromone components have been identified by researchers and are viewed as potential means for mass trapping, disruption of mating communication, monitoring and surveying [5]. In addition, *Bacillus thuringiensis*, Nuclearpolyhedrosis viruses (NPVs), and *Beauveria bassiana* have shown variable levels of efficacy on the pest [6–8]. Although all of these studies about management of *Spodoptera exigua*, economic losses caused by the pest still continues.

Although variety of bacterial phyla including *Firmicules*, *Actinobacteria*, *Bacteriodetes*, *Proteobacteria*, *Clostridiales*, etc. have been

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Table 1
Oligonucleotide primers used for screening of partial *cry* type genes.

Name	Amplicon	Primer sequences (5' - > 3′)	T_m (°C)
Un1	Partial *cry1* gene (277 bp)	CATGATTCATGCGGCAGATAAAC (d) TTGTGACACTTCTGCTTCCCATT (r)	55
Un2	Partial *cry2* gene (701 bp)	GTTATTCTTAATGCAGATGAATGGG (d) CGGATAAAATAATCTGGGAAATAGT (r)	52
Un3	Partial *cry3* gene (604bp)	CGTTATCGCAGAGAGATGACATTAAC (d) CATCTGTTGTTTCTGGAGGCAAT (r)	54
Un4	Partial *cry4* gene (439 bp)	GCATATGATGTAGCGAAACAAGCC (d) GCGTGACATACCCATTTCCAGGTCC (r)	59
Un5	Partial *cry5* gene (474 bp)	TTACGTAAATTGGTCAATCAAGCAAA (d) AAGACCAAATTCAATACCAGGGTT (r)	52
Un7-8	Partial *cry7*gene (420 bp)	AAGCAGTGAATGCCTTGTTTAC (d) CTTCTAAACCTTGACTACTT (r)	49
Un9	Partial *cry9* gene (359 bp)	CGGTGTTACTATTAGCGAGGGCGG (d) GTTTGAGCCGCTTCACAGCAATCC (r)	60
Un11	Partial *cry11* gene (305 bp)	TTCCAACCCAACTTTCAAGC (d) AGCTATGGCCTAAGGGGAAA (r)	51

d: Forward primer; r: Reverse primer.

isolated from lepidopteran larvae [9–12], the role of the gut bacteria has not been fully clarified. The relationships between gut microbiota and their host may range from symbiotic to pathogenic and from facultative to obligate [13]. Understanding the role of the gut microbiota is an important step toward the process of using bacteria in the microbial control of the pest. Nowadays, entomopathogenic bacteria isolated from gut biota of various agricultural pests have been used as microbial pesticide to control the damage levels of noxious insects [14,15].

In this respect, we aimed the determination of the culturable gut microbiota of *Spodoptera exigua* for finding more virulent and safe bacterial control agent against the pest. We isolated 15 bacteria from the *S. exigua* larvae and identified them in detail using phenotypic, genotypic, and phylogenetic techniques. Furthermore, we tested larvicidal potency of its microbiota, and we found that one of them is highly pathogenic to the larvae. This pathogenic isolate could be developed as a bacterial biopesticide that could be used to reduce economic losses caused by *S. exigua*.

2. Materials and methods

2.1. Isolation of culturable bacteria

Third instar larvae of *Spodoptera exigua* were collected from infested corn fields in the vicinity of Adana, Turkey. To isolate the gut bacteria, thirty randomly selected larvae were selected and starved for 24 h. The starved larvae were surface disinfected with 70% ethanol for 5 min followed by washing with sterile distilled water to remove the disinfectant [16]. Larvae were dissected in 10 mM sterile phosphate-buffered saline (PBS) inside a sterile laminar flow hood using dissection scissors and fine-tipped forceps. The excised larval guts were homogenized in nutrient broth medium using a homogenizer. The homogenized suspension was filtered into sterile tubes to remove larval debris. Afterward, 10, 25 and 50 µL of filtered suspension were spread on nutrient agar plates. The plates were incubated at 30 °C and observed daily for appearance of morphological distinct colonies up to 72 h. The colonies were differentiated based on their size, color, and morphology, and a single representative isolate of each morphotype transferred to new plates. Pure cultures of bacterial isolates were prepared and stocked in 20% glycerol at -80 °C at Karadeniz Technical University, Department of Biology, Laboratory of Microbiology, Trabzon, Turkey.

2.2. Phenotypic identification of the isolates

Macroscopic and microscopic characterization of the isolates were performed and evaluated according to Bergey's Manual of Systematic Bacteriology 1 and 2 [17,18]. Colony morphology of bacterial isolates, plated out on nutrient agar plates, was inspected by stereomicroscope. The cell shapes and mobility of the bacterial isolates were also determined using a light microscope at 1000 × magnification. Gram staining and endospore staining were performed according to the procedure described by Claus [19] and Reynolds et al. [20]. Biochemical characterization of the bacterial isolates was determined via VITEK-2 system that is an automated microbial identification system employing tests of carbon source, enzymatic activities and resistance to antibiotics [21].

2.3. Genotypic and phylogenic identification of the isolates

Genomic DNA extracted from bacteria using DNA purification kit (Promega, France) according to the manufacturer's protocols, was stored at -20 °C until use. The 16S rRNA genes of the bacterial isolates were amplified using PCR with universal bacterial forward primer UNI16S-F (5′-ATTCTAGAGTTTGATCATGGCTCA-3′) and reverse primer UNI16S-R (5′-ATGGTACCGTGTGACGGGCGGTGTGTA-3′) [22]. PCR amplification was performed with the following program: 95 °C for 30 s; 30 cycles at 95 °C for 30 s, 50 °C for 30 s and 68 °C for 1 min, and a final extension at 68 °C for 5 min. PCR products were analysed by electrophoresis on 1.0% agarose gel and visualized under UV light. The

Table 2
Phenotypic characteristics of bacterial isolates.

Isolates	Macroscopic Characteristics			Microscopic Characteristics			
	Colony color	Colony shape	Growth in NB	Cell shape	Gram staining	Spore staining	Motility
Se1	Cream	Rough	Turbid	Bacillus	+	+	+
Se2	White	Smooth	Turbid	Bacillus	+	+	+
Se3	White	Smooth	Turbid	Bacillus	–	–	+
Se4	Cream	Smooth	Turbid	Bacillus	+	+	+
Se5	White	Smooth	Turbid	Coccos	+	–	–
Se6	Cream	Smooth	Turbid	Bacillus	–	–	–
Se7	Yellow	Smooth	Turbid	Bacillus	–	–	–
Se8	Cream	Rough	Turbid	Bacillus	+	+	+
Se9	Red	Smooth	Turbid	Bacillus	–	+	+
Se10	Cream	Smooth	Turbid	Bacillus	+	+	+
Se11	Cream	Smooth	Turbid	Bacillus	+	+	+
Se12	White	Smooth	Turbid	Bacillus	–	–	+
Se13	Cream	Smooth	Turbid	Bacillus	+	+	+
Se14	Cream	Smooth	Turbid	Bacillus	+	+	+
Se15	Dark cream	Smooth	Turbid	Bacillus	+	+	+