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ARDAHAN ESKİ

FİLİZ ÖZKAN ÇAKICI

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ZİHNİ DEMİRBAĞ

See next page for additional authors

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Identification and pathogenicity of bacteria in the Mediterranean corn borer *Sesamia nonagrioides* Lefebvre (Lepidoptera: Noctuidae)

Ardahan ESKİ[1], Filiz ÖZKAN ÇAKICI[1], Mustafa GÜLLÜ[2], Hacer MURATOĞLU[3], Zihni DEMİRBAĞ[1], İsmail DEMİR[1,*]

[1]Department of Biology, Faculty of Sciences, Karadeniz Technical University, Trabzon, Turkey
[2]Department of Entomology, Biological Control Research Station, Adana, Turkey
[3]Department of Molecular Biology and Genetics, Faculty of Sciences, Karadeniz Technical University, Trabzon, Turkey

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Abstract: *Sesamia nonagrioides* (Lep.: Noctuidae) is one of the most serious pests of corn in Turkey and other Mediterranean countries. Although various cultural, chemical, and biological methods are used to control this pest, its damage still continues in all Mediterranean countries. In this study, to find an effective bacterium that can be used as a biocontrol agent against *S. nonagrioides*, we isolated 15 bacteria from *S. nonagrioides* larvae and evaluated the larvicidal potency of all isolates on the pest. According to their morphological, physiological, biochemical, and molecular properties, the isolates were identified as *Achromobacter insolitus* (Sn1), *Morganella morganii* (Sn2), *Klebsiella pneumoniae* (Sn3), *Citrobacter freundii* (Sn4), *Arthrobacter protophormiae* (Sn5), *Chryseobacterium indologenes* (Sn6), *Bacillus thuringiensis* (Sn7), *Bacillus safensis* (Sn8), *Bacillus thuringiensis* (Sn9), *Bacillus thuringiensis* (Sn10), *Klebsiella pneumoniae* (Sn11), *Staphylococcus sciuri* (Sn12), *Enterobacter kobei* (Sn13), *Serratia marcescens* (Sn14), and *Microbacterium arborescens* (Sn15). The results of the larvicidal activities of these isolates indicated that the mortality value obtained from all treatments varied from 25% to 93%, reaching 93% with *B. thuringiensis* (Sn10) for the third-instar larvae within 10 days of the application of 1.89×10^9 cfu/mL bacterial concentration at 25 °C in laboratory conditions. However, the dose-response experiments showed that increasing the concentration of bacteria gradually increased larval mortality, which reached 100% with a 2-fold concentration of Sn10. The findings of this study indicate that this isolate appears to be a promising biocontrol agent for use against *S. nonagrioides*.

Key words: *Sesamia nonagrioides*, bacterial flora, *Bacillus thuringiensis*, biocontrol

1. Introduction

Corn is the largest grown cereal crop in the world with doubled grain yield per unit area compared to wheat and barley. Although corn production holds second place in Mediterranean agricultural production, there has been a sharp decline in this value due to infestation by the Mediterranean corn borer (Özcan, 2009). The Mediterranean corn borer, *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae), is a major multivoltine pest of maize in Mediterranean countries (Tsitsipis, 1988; Alexandri and Tsitsipis, 1990). *S. nonagrioides* is a polyphagous species with a fairly wide range of host plants, including corn, sorghum, millet, rice, sugar cane, grasses, melon, asparagus, palms, and banana (Uygun and Kayapınar, 1993; Sannino et al., 2004). The population levels of this species, which has considerable potential to establish itself in an area and become abundant, may therefore depend on the abundance of these hosts (Eizaguirre and Fantinou, 2012). If insecticides are not applied, losses could rise to 100% in late and second crop production (Alexandri and Tsitsipis, 1990).

Application of chemical insecticides against this harmful insect is recommended 2–3 times per growing season. However, yield losses could rise to 30% during seasons with severe insect outbreaks, even after 4–5 insecticide applications (Özcan, 2009). As well as causing significant maize crop losses, the insects reduce the nitrogen/protein content of grain by tunneling into the stem and cobs, where they are likely to interfere with the uptake of plant nutrients (Bayram, 2003). Furthermore, mycotoxigenic fungi associated with the pest often invade wound sites and can greatly depreciate the crop value (Avantaggiato et al., 2003). Although several control methods have been previously applied to this pest, damage is still pervasive. Chemical insecticides have been frequently used against it. While chemicals decrease pest population, they cause major threats to the environment and human health. Mechanical, cultural, and biological

* Correspondence: idemir@ktu.edu.tr

methods have also been used to control this pest. For example, it has been found that a natural enemy of *S. nonagrioides* is *Telenomus busseolae*, an egg parasitoid (Sertkaya and Kornoşor, 2003). This does not offer control and is not currently used in any biocontrol programs.

The current methods are not adequate to control *S. nonagrioides*. The control of this pest is faced with major problems considering the epidemics it causes every year. In recent years, an alternative control method has appeared in the place of chemical control, which involves organic farming studies in corn fields. Microbial control is distinguished by a delimitation of natural enemies and a decrease in the population of the pest. Thus there is still need to find microbes that can be used to control *S. nonagrioides*.

The use of microbial agents against harmful insects is the most accepted approach in the world, as they limit pest infestations. Microorganisms used in pest control are not essentially toxic and pathogenic to nontarget organisms (Uygun et al., 2010). Microbial agents (entomopathogenic viruses, bacteria, fungi, nematodes, and protozoans) and their products are successfully applied in different parts of the world. Considering environmental and biological conditions, the most appropriate microbial control agent against *S. nonagrioides*, which causes crop loss in Turkey, should originate from entomopathogenic bacteria. To our knowledge, no study regarding utilization of entomopathogenic bacteria in the microbial control of *S. nonagrioides* has been conducted until now.

In the present study, we focus on the determination of the culturable bacterial flora of *Sesamia nonagrioides* in order to find virulent and safe biocontrol agents against the pest and to provide suitable symbiotic bacteria that can be genetically modified to express poisonous proteins to kill this or other pests. For this purpose, we isolated 15 bacteria from *S. nonagrioides* larvae and identified them in detail using morphological, biochemical, physiological, and molecular techniques. Additionally, we tested the larvicidal potency of all bacterial isolates against the larvae of the pest, and we found that 2 of them are highly toxic to the larvae. The isolation and determination of these pathogenic strains could be important for the future development of biotechnological strategies aimed at reducing the economic losses caused by *S. nonagrioides*.

2. Materials and methods

2.1. Collection of larvae

S. nonagrioides larvae were collected from infested corn fields in the area of Adana, Turkey. Corn stems containing *S. nonagrioides* larvae were collected during the 2011/2012 seasons. Collected stems were carefully dissected and larvae were removed and put into plastic boxes with perforated covers to permit airflow. Fresh corn stems were provided as food and the boxes were immediately transported to the laboratory. Following transportation, we continued to feed healthy larvae with fresh stems at room temperature under a 12:12 photoperiod for 1 week. Finally, larvae were used for bacterial isolation.

2.2. Isolation of bacteria

Bacterial isolation was performed from larvae. Twenty field-collected larvae were surface-sterilized with 70% ethanol for 5 min (Poinar and Thomas, 1978) and washed 3 times in sterile distilled water. The larval bodies were homogenized in nutrient broth using a glass tissue grinder, and the homogenate was filtered through 2 layers of cheese muslin into sterile tubes to remove larval debris. Afterwards, 10, 25, and 50 µL of larval extracts were plated on nutrient agar and incubated at 30 °C for 2–3 days. The remaining mixtures were incubated at 30 °C for 3–4 h to enrich the number of bacteria that had low concentration. From these mixtures, 10, 25, and 50 µL were also plated on nutrient agar and incubated at 30 °C for 2–3 days. Finally, the incubated larval suspensions were heated in a water bath at 80 °C for 10 min to eliminate nonspore-forming bacteria (Ohba and Aizawa, 1986). After heat inactivation, 10, 25, and 50 µL of the heated suspensions were plated on nutrient agar and incubated at 30 °C for 2–3 days. Isolates were distinguished based on colony color and morphology. Pure cultures of bacterial colonies were prepared and were stocked in 20% glycerol at –80 °C in the Microbiology Laboratory, Department of Biology, Faculty of Sciences, Karadeniz Technical University, Trabzon, Turkey. Bacterial cultures were identified according to their morphology, nutritional features, and biochemical and molecular characteristics.

2.3. Phenotypic and biochemical identification of the isolates

Phenotypic and biochemical characterization of isolates was conducted and evaluated according to *Bergey's Manual of Systematic Bacteriology*, Vols. 1 and 2 (Krieg and Holt, 1986; Sneath et al., 1986). Colony morphologies of bacterial isolates, plated out on nutrient agar plates, were inspected by direct observation and under a stereomicroscope. The shape of the bacterial isolates was also determined using a light microscope at 1000× magnification. Gram staining was performed according to the procedure described by Claus (1992). Endospore staining was carried out with the method of Prescott et al. (1996). The API 20E, API 50CH, and VITEK-2 systems were also used for biochemical characterization of the bacterial isolates. API test results were evaluated using IdBactv. 1.1 software by G Kronvall, with Matrix for API20E and API 50CHB from bioMerieux, France. VITEK-2 is an automated microbial identification system that provides highly accurate and reproducible results with its colorimetric cards and associated hardware and software advances.

2.4. 16S rRNA gene sequencing

Partial sequencing of the 16S rRNA gene was used to confirm isolate identification. Total genomic DNAs of bacterial isolates were extracted according to the standard protocol of Sambrook et al. (1989) and stored at –20 °C until use.

PCR amplification of 16S rRNA genes of the bacterial isolates was performed with universal primers. UNI16S-F (5'-ATTCTAGAGTTTGATCATGGCTCA-3' and UNI16S-R (5'-ATGGTACCGTGTGACGGGCGGTGT-GTA-3') primers were used as forward and reverse primers, respectively (Weisburg et al., 1991). PCR amplification was carried out in a thermocycler (Eppendorf, Mastercycler Gradient, Hamburg, Germany) for 36 reaction cycles. Reactions were routinely performed in 50 µL: 1.5 µL of 10 mM dNTP mix, 1.5 µL of 10 pmol each of the opposing amplification primers, 0.5 µL of 5 U/µL of Taq DNA polymerase, 3 µL of 2.5 mM $MgCl_2$, 10 µL of 5X reaction buffer, 2 µL of 250 ng/µL genomic DNA, and 32 µL dH_2O. The PCR amplification program of 16S rRNA primers included an initial denaturation of the template DNA at 94 °C for 2 min followed by 35 cycles with a denaturation step at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. PCR products were separated on 1.0% agarose gels, stained with ethidium bromide, and viewed under UV light. After confirming the PCR products, amplified fragments were cloned into the pGEM-T Easy Vector (Promega Co., USA). The ligation mixture was transformed into the *Escherichia coli* JM101 strain (Sambrook et al., 1989). After amplification, plasmid DNA samples were digested by *Eco*RI restriction enzyme (Promega) for confirming, and confirmed plasmids were sent to MACROGEN (the Netherlands) for sequencing.

The obtained sequences were used to perform BLAST searches (Altschul et al., 1990) using the NCBI GenBank database. Additionally, they were used for phylogenetic analysis to confirm isolates identification.

2.5. *Cry* gene contents and crystal protein profiles of *Bacillus thuringiensis* (Sn10)

Based on identification tests, one bacterial isolate, Sn10, was identified as *Bacillus thuringiensis*. To amplify the *cry* genes of Sn10, which has the highest insecticidal activity among bacterial isolates, genomic DNA was extracted from this bacterial culture using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. PCR reaction was performed with 4 pairs of universal primers for the *cry*1, *cry*2, *cry*3, and *cry*4 genes as described by Ben-Dov et al. (1997) (Table 1). PCR amplifications were performed in a total volume of 50 µL, which included 10 µL of 5X GoTaq flexi buffer, 3 µL of 2.5 mM $MgCl_2$, 1 µL of 10 mM dNTP mix, 1.5 µL each of the opposing amplification primers (10 mM), 2.5 units of Taq DNA polymerase (Promega), and 50 ng of genomic DNA. The final volume was completed with ddH_2O. PCR reaction mixtures were processed for 30 PCR cycles (94 °C for 1 min, 48–66 °C for 30 s, and 72 °C for 1 min). PCR-amplified fragments were purified with a NucleoSpin purification kit (Macherey-Nagel) and ligated into pGEM-T Easy Vector (Promega). The ligation mixture was transformed to *E. coli* JM101 strain. After amplifications, plasmid DNA was digested by *Eco*RI restriction enzyme for confirmation, and the confirmed plasmid was sequenced. The sequence obtained was used to perform BLAST searches using the NCBI GenBank database.

To determine the presence of Cry proteins, proteins were extracted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Sn10 was grown in nutrient agar medium and incubated for 7 days at 30 °C. Later, the spore-crystal mixture was collected from the surface of the medium. The spore-crystal mixture was resuspended in 1 M cold NaCl and centrifuged at 12,000 rpm for 5 min. The pellet was washed twice in sterile dH_2O and resuspended with sterile dH_2O. Protein concentrations were determined by Bradford assay (1970) and analyzed

Table 1. The partial primers for the identification of *cry* genes.

Primer name	Primer sequence	Tm (°C)	Reference
Cry1R	5'-TTGTGACACTTCTGCTTCCCATT-3'	66	Ben-Dov et al., 1997
Cry1F	5'-CATGATTCATGCGGCAGATAAAC-3'		
Cry2R	5'-CGGATAAAATAATCTGGGAAATAGT-3'	61	
Cry2F	5'-GTTATTCTTAATGCAGATGAATGGG-3'		
Cry3R	5'-CATCTGTTGTTTCTGGAGGCAAT-3'	55	
Cry3F	5'-CGTATACGCAGAGAGATGACATTAAC-3'		
Cry4R	5'-GCGTGACATACCCATTTCCAGGTCC-3'	48	
Cry4F	5'-GCATATGATGTAGCGAAACAAGCC-3'		

in 10% SDS-PAGE as described by Laemmli (1970). Silver staining was used to detect proteins after electrophoretic separation on polyacrylamide gel, and protein molecular weight was estimated by comparison to protein molecular weight standards (Novex prestained protein marker).

2.6. Phylogeny

Nucleotide sequences of 16S rRNA genes and *cry* genes were edited with BioEdit and aligned by ClustalW (Hall, 1999). Phylogenetic analysis was performed using the neighbor-joining (NJ) method, as carried out by MEGA 5.0 software (Tamura et al., 2011). The NJ analysis was based on the Kimura 2-parameter test. Alignment gaps were treated as missing data. The reliability of the phylograms was tested by bootstrap analysis with 1000 replicates using MEGA 5.0.

2.7. Bioassays

Bacterial isolates were streaked on nutrient agar plates to obtain single colonies for each isolate. The obtained single colonies were inoculated into nutrient broth medium and incubated overnight at 30 °C. Several isolates were incubated at 30 °C for 2 days due to their slow growth. After incubation, bacterial density was measured at OD_{600} (optical density) and adjusted to 1.8×10^9 cfu/mL (Ben-Dov et al., 1995). Five milliliters of this culture was centrifuged at 3000 rpm for 10 min. Afterwards, the pellet was resuspended in 5 mL of sterile phosphate-buffered saline and used in bioassays.

The susceptibility of *S. nonagrioides* larvae to each bacterial isolate was tested under laboratory conditions. The third-instar of healthy larvae obtained from corn fields in the vicinity of Adana were randomly selected and used in bioassays. Fresh corn stems were used as diet in infections. One milliliter of bacterial suspension for each isolate, prepared as described above, was saturated on corn stems and placed in individual plastic boxes, each containing a single bacterial isolate. Thirty third-instar larvae were placed on the diet in containers and kept at 30 ± 2 °C and 60% relative humidity on a 12:12 photoperiod (Mitchell and Smith, 1985). Larvae mortality was recorded every 24 h and all dead larvae were removed from the containers. All bioassays were repeated 3 times on different occasions.

Three isolates (Sn8, Sn10, and Sn14) that had high mortality against *S. nonagrioides* were used in dose-response experiments. Three different bacterial concentrations (0.9×10^9 cfu/mL, 1.8×10^9 cfu/mL, and 3.6×10^9 cfu/mL, based on OD_{600} values) of these isolates were used in the dose-response experiments, and bioassays were performed as described earlier.

2.8. Data analysis

Percent mortality was corrected according to Abbott's formula (Abbott, 1925). The data were subjected to ANOVA and subsequently to least significant difference (LSD) multiple comparison tests to compare test isolates with each other and the control group. The lethal concentrations (LC_{50} and LC_{95}) were estimated by probit analysis. All analyses were performed using SPSS 20.0 software.

3. Results

A total of 15 bacteria were isolated from *S. nonagrioides* larvae and were identified using a number of methods mentioned earlier. All isolates were identified at the species level. Colonies were observed in different colors on nutrient agar. The colony shapes of isolates Sn7, Sn9, and Sn10 were rough; the others were smooth. While only one isolate (Sn12) was a coccus, the others were bacilli. Seven isolates (Sn5, Sn7, Sn8, Sn9, Sn10, and Sn15) were gram-positive; the rest were gram-negative. Four gram-positive bacteria (Sn7–Sn10) were spore-forming (Table 2).

Table 2. The morphological characteristics of the bacterial isolates of *S. nonagrioides*.

Isolates	Colony color	Colony shape	Motility	Growth in NB	Cell shape	Gram staining	Spore staining
Sn1	Cream	Smooth	+	Turbid	Bacillus	–	–
Sn2	Light cream	Smooth	+	Turbid	Bacillus	–	–
Sn3	Cream	Smooth	–	Turbid	Bacillus	–	–
Sn4	Light cream	Smooth	+	Turbid	Bacillus	–	–
Sn5	Yellow	Smooth	–	Turbid	Bacillus	+	–
Sn6	Yellow	Smooth	+	Turbid	Bacillus	–	–
Sn7	Cream	Rough	+	Turbid	Bacillus	+	+
Sn8	Light cream	Smooth	+	Turbid	Bacillus	+	+
Sn9	Cream	Rough	+	Turbid	Bacillus	+	+
Sn10	Cream	Rough	+	Turbid	Bacillus	+	+
Sn11	Cream	Smooth	+	Turbid	Bacillus	–	–
Sn12	Dark cream	Smooth	–	Turbid	Coccus	+	–
Sn13	Dark cream	Smooth	+	Turbid	Bacillus	–	–
Sn14	Cream	Smooth	+	Turbid	Bacillus	–	–
Sn15	Orange	Smooth	+	Turbid	Bacillus	+	–

NB: Nutrient broth.

All isolates were grown in nutrient broth, adjusted to pH 8. While Sn9 and Sn11 grew at pH 3, the others did not grow. All isolates were grown in nutrient broth containing 3% NaCl at 20 °C and 30 °C. All spore-forming bacteria and Sn2 and Sn13 (a gram-negative bacterium) were also grown at 50 °C (Table 3).

The biochemical characteristics of the bacterial isolates varied depending on the isolate. While the API 20E test was used for gram-negative bacteria, the API 50CH test was used for gram-positive bacteria. The results of API tests are shown in Tables 4 and 5. In order to support the results of the API tests, VITEK-2 tests were performed and the results are displayed in Tables 6–8. The results of this test also varied depending on the isolate.

We also sequenced approximately 1.350 bp of 16S rRNA gene for each isolate to confirm molecular identification of isolates. The similarities of each isolate to known species are shown in Table 9. Based on identification tests and sequencing analysis, the isolates were identified as *Achromobacter insolitus* (Sn1), *Morganella morganii* (Sn2), *Klebsiella pneumoniae* (Sn3), *Citrobacter freundii* (Sn4), *Arthrobacter protophormiae* (Sn5), *Chryseobacterium indologenes* (Sn6), *Bacillus thuringiensis* (Sn7), *Bacillus safensis* (Sn8), *Bacillus thuringiensis* (Sn9), *Bacillus thuringiensis* (Sn10), *Klebsiella pneumoniae* (Sn11), *Staphylococcus sciuri* (Sn12), *Enterobacter kobei* (Sn13), *Serratia marcescens* (Sn14), and *Microbacterium arborescens* (Sn15). Phylogenetic analysis of 16S rRNA genes also supports this identification (Figure 1).

Following identification of all isolates, we screened *cry* genes from *Bacillus thuringiensis* (Sn10), which had the highest insecticidal activity. We observed that Sn10 contains both *cry*1 and *cry*2 genes. We obtained approximately 400 bp of *cry*1 and 700 bp of *cry*2 genes by PCR amplification. The comparison of homology of the nucleotide sequence in the NCBI GenBank database using the BLAST program showed that they shared the highest homology (99%) with other known *cry*1Ac and *cry*2Ab genes. Phylogenetic analysis of *cry*1 and *cry*2 genes also supported this identification (Figures 2 and 3). SDS-PAGE was performed to determine the presence of Cry proteins in the total protein extract of *Bacillus thuringiensis* (Sn10), and the result clearly indicates the presence of the proteins (Figure 4). The detection of protein bands of ~130 kDa and ~65 kDa in protein extracts belonging to the *cry*1 and *cry*2 genes is in agreement with the PCR results.

In addition, we tested the insecticidal activity of bacterial isolates that were isolated from *S. nonagrioides* larvae against the third-instar larvae of the pest as a possible biocontrol agent. Although most isolates displayed different mortality rates on the pest, Sn3, Sn4, Sn13, and Sn15 caused a similar mortality effect. Sn7, Sn9, and Sn11 also showed a similar mortality effect. Other isolates caused statistically different mortality ($F = 62.624$; $df = 15$; $P < 0.05$). While the highest mortality was caused by Sn10 at 93% within 10 days of 12:12 photoperiods in laboratory conditions, the other mortalities ranged from 25% to 80% (Figure 5).

Table 3. The physiological characteristics of the bacterial isolates of *S. nonagrioides*.

Isolate	pH						NaCl tolerance (%)			Temperature (°C)				
	3	4	5	6	8	11	3%	5%	7%	20	30	37	45	50
Sn1	−	−	+	+	+	+	+	−	−	+	+	+	+	−
Sn2	−	−	+	+	+	+	+	+	+	+	+	+	+	+
Sn3	−	+	+	+	+	+	+	+	+	+	+	+	+	−
Sn4	−	+	+	+	+	+	+	+	+	+	+	+	+	−
Sn5	−	−	+	+	+	+	+	+	+	+	+	+	+	−
Sn6	−	−	−	+	+	−	+	−	−	+	+	−	−	−
Sn7	−	−	+	+	+	+	+	+	−	+	+	+	+	+
Sn8	−	−	+	+	+	+	+	+	+	+	+	+	+	+
Sn9	+	+	+	+	+	+	+	−	−	+	+	+	+	+
Sn10	−	−	−	+	+	+	+	−	−	+	+	+	+	+
Sn11	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Sn12	−	−	+	+	+	+	+	+	+	+	+	+	+	−
Sn13	−	+	+	+	+	+	+	+	+	+	+	+	+	+
Sn14	−	+	+	+	+	+	+	+	+	+	+	+	+	−
Sn15	−	−	−	−	+	+	+	+	+	+	+	−	−	−

Table 4. The biochemical characteristics of the gram-positive bacterial isolates based on API 50 CHB bacterial identification system.

Tests	Isolates						
	Sn5	Sn7	Sn8	Sn9	Sn10	Sn12	Sn15
Glycerol	–	–	–	–	–	–	–
Erythritol	–	–	+	–	+	–	–
D-Arabinose	–	–	–	–	–	–	–
L-Arabinose	–	–	–	–	–	–	–
D-Ribose	–	–	+	–	–	–	–
D-Xylose	+	+	+	–	+	–	–
L-Xylose	–	–	+	–	–	–	–
D-Adonitol	–	–	+	–	–	–	+
Methyl-β-D-xylopyranoside	–	–	+	–	–	–	+
D-Galactose	–	–	+	–	–	–	+
D-Glucose	–	–	+	–	–	–	–
D-Fructose	+	+	+	+	+	+	+
D-Mannose	+	+	–	+	+	+	+
L-Sorbose	–	–	–	–	+	–	–
L-Rhamnose	–	–	–	–	–	–	–
Dulcitol	–	–	+	–	–	–	–
Inositol	–	–	–	–	–	–	–
D-Mannitol	–	–	+	–	–	–	–
D-Sorbitol	–	–	+	–	–	–	–
Methyl-αD-mannopyranoside	+	–	+	–	–	–	–
Methyl-αD-glucopyranoside	+	–	–	–	+	–	–
N-Acetylglucosamine	+	–	+	–	+	–	–
Amygdalin	+	+	+	+	+	+	+
Arbutin	–	–	+	–	–	–	–
Esculin-ferric citrate	–	–	–	+	+	+	+
Salicin	–	–	+	+	+	+	+
D-Cellobiose	+	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+	+
D-Lactose (bovine origin)	+	+	+	+	+	+	+
D-Melibiose	+	+	–	–	–	–	–
D-Saccharose (sucrose)	+		–	–	–	–	–
D-Trehalose	–	–	+	+	+	+	+
Inulin	–	–	–	+	+	+	+
D-Melezitose	–	–	–	–	+	–	–
D-Raffinose	+	+	+	–	–	–	–
Glycogen	+	–	+	+	+	+	+
Xylitol	–	–	+	+	+	+	+
Gentiobiose	+	+	+	–	+	–	–
D-Turanose	–	–	+	–	+	–	–
D-Lyxose	–	–	–	–	–	–	–
D-Tagatose	+	–	+	–	–	–	–
D-Fucose	+	–	+	–	–	–	–
L-Fucose	+	–	–	–	–	–	–
D-Arabitol	–	–	+	–	–	–	–
L-Arabitol	–	–	+	–	–	–	–
Potassium gluconate	+	–	+	–	–	–	–
Potassium 2-ketogluconate	+	–	+	–	–	–	–
Potassium 5-ketogluconate	–	–	+	–	–	+	+

Table 5. The biochemical characteristics of the bacterial isolates based on API 20E bacterial identification system.

Substrates	Activity	Sn 1	Sn 2	Sn 3	Sn 4	Sn 5	Sn 6	Sn 7	Sn 8	Sn 9	Sn 10	Sn 11	Sn 12	Sn 13	Sn 14	Sn 15
ONPG	β-Galactosidase	+	−	+	+	+	+	−	−	+	+	+	−	+	+	+
Arginine	Arginine dihydrolase	−	−	−	−	−	−	+	+	+	+	−	+	+	−	+
Lysine	Lysine decarboxylase	−	−	+	−	+	+	−	+	+	+	+	+	+	+	+
Ornithine	Ornithine decarboxylase	−	+	−	−	−	+	−	+	+	−	−	+	+	+	−
Citrate	Citrate utilization	−	−	+	+	+	+	+	+	+	−	+	+	+	+	+
Na thiosulfate	H2S production	−	−	−	+	−	+	−	−	+	−	−	+	+	−	+
Urea	Urea hydrolysis	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−
Tryptophan	Deaminase	−	+	−	−	−	+	−	+	−	+	−	−	+	−	+
Tryptophan	Indole production	+	+	−	−	−	+	−	+	−	+	−	+	+	−	+
Acetoin production	Colorless	−	+	+	+	+	+	−	+	+	−	+	+	+	+	−
Charcoal gelatin	Gelatinase	+	−	−	−	−	+	+	+	+	−	−	+	−	+	−
Glucose	Fermentation/oxidation	−	+	+	+	−	+	−	+	+	−	+	−	−	+	+
Mannitol	Fermentation/oxidation	−	−	+	+	+	+	−	+	+	+	+	−	+	+	+
Inositol	Fermentation/oxidation	−	−	+	−	+	+	−	−	+	+	−	+	+	+	−
Sorbitol	Fermentation/oxidation	−	−	+	+	+	+	−	+	+	+	+	+	+	+	+
Rhamnose	Fermentation/oxidation	−	−	+	+	+	−	−	+	+	+	+	+	+	−	+
Sucrose	Fermentation/oxidation	−	−	+	+	+	+	−	+	−	+	+	+	+	+	+
Melibiose	Fermentation/oxidation	−	−	+	+	+	+	−	−	+	+	+	+	+	+	−
Amygdalin	Fermentation/oxidation	−	−	+	−	+	+	−	+	−	+	+	+	+	+	+
Arabinose	Fermentation/oxidation	−	−	+	+	+	+	−	+	+	+	+	+	+	−	+

Finally, 3 effective isolates (Sn8, Sn10, and Sn14) were tested against *S. nonagrioides* in dose-response experiments. Isolate Sn10, identified as *B. thuringiensis*, had 100% larval mortality with 2-fold concentration (3.7×10^9 cfu/mL) ($F = 248.34$; $df = 9$; $P < 0.05$). Sn8, Sn10, and Sn14 showed statistically similar mortality rates at 0.9×10^9 cfu/mL concentration. However, isolates caused statistically different mortality at 1.8×10^9 cfu/mL and 3.7×10^9 cfu/mL concentrations (Figure 6). Lethal concentrations (LC_{50} and LC_{95}) of these isolates were also calculated (Table 10). LC_{50} values were calculated as 0.258×10^9, 0.596×10^9, and 0.0002×10^9 cfu/mL for Sn8, Sn10, and Sn14, respectively.

4. Discussion

The chemical insecticides used against insect pests have caused unprecedented harm to the environment. Growing concern about harmful effects has necessitated a change in strategy to manage insect pests in an ecologically sustainable manner. These concerns prompted scientists to seek biopesticides such as microbial pesticides. Entomopathogenic microorganisms are an attractive, effective, and environmentally safe alternative to the control of many noxious pest species in both agriculture and forestry because they are safe for animals, plants, and environment. So far, many pathogens have been isolated from pests, and their insecticidal effects on agricultural pests have been determined (Kati et al., 2010; Sevim et al., 2010a; Tanyeli et al., 2010; Danismazoglu et al., 2012; Demir et al., 2013; Demirci et al., 2013). Some entomopathogenic agents have been improved and are still being used as biopesticides in pest management systems (Charles et al., 2000; Grewal et al., 2005; Lacey and Kaya, 2007).

In this study, to find a microorganisms that can be utilized against *S. nonagrioides*, we focused on the determination of the culturable bacterial flora of *S. nonagrioides* and on the investigation of the biocontrol potential of the isolated bacterial species against the pest. Fifteen bacterial isolates (Sn1–Sn15) were determined based on colony morphology among the total number of bacterial isolates and were characterized based on morphological, nutritional, physiological, and biochemical characteristics (API and VITEK-2) and 16S rRNA. The phenotypic features were compared to *Bergey's Manual of Systematic Bacteriology*.

Bacteria isolated from *S. nonagrioides* have been isolated from various insect species before (Inglis et al., 2000; Dugas et al., 2001; İnce at al., 2008; Sevim et al., 2010b; Ademolu and Idowu, 2011). However, for the

Table 6. The biochemical characteristics of gram-negative bacterial isolates based on VITEK-2 bacterial identification system.

Tests	Isolates							
	Sn1	Sn2	Sn3	Sn4	Sn6	Sn11	Sn13	Sn14
Ala-Phe-Pro arylamidase	−	−	−	−	+	−	−	−
Adonitol	−	−	+	−	−	+	+	+
L-Pyrrolidonyl-arylamidase	−	−	+	+	+	+	+	+
L-Arabitol	−	−	−	−	−	−	−	+
D-Cellobiose	−	−	+	−	−	+	+	−
β-Galactosidase	−	−	+	+	−	+	+	−
H_2S production	+	+	−	+	−	−	−	−
β-N-Acetyl-glucosaminidase	−	−	+	−	−	+	+	+
Glutamyl arylamidase pNA	+	−	−	−	+	−	+	−
D-Glucose	+	+	+	+	−	+	+	+
γ-Glutamyl-transferase	+	+	+	+	+	+	+	−
Fermentation/glucose	+	+	+	+	−	+	+	+
β-Glucosidase	+	−	+	−	+	+	−	−
D-Maltose	+	−	+	+	−	+	+	+
D-Mannitol	−	−	+	+	−	+	−	+
D-Mannose	+	+	+	+	−	+	+	−
β-Xylosidase	+	−	+	−	−	+	+	+
β-Alanine arylamidase pNA	+	−	+	−	−	+	−	+
L-Proline arylamidase	−	+	−	+	+	−	+	+
Lipase	+	−	+	−	+	+	+	−
Palatinose	+	−	−	−	−	−	−	−
Tyrosine arylamidase	−	+	+	+	+	+	+	+
Urease	+	+	+	−	−	+	−	−
D-Sorbitol	−	−	+	+	−	+	+	+
Saccharose/sucrose	−	−	+	+	−	+	+	+
D-Tagatose	−	−	−	−	−	−	+	−
D-Trehalose	+	−	+	+	−	+	−	+
Citrate (sodium)	−	−	+	+	−	+	+	+
Malonate	−	−	+	−	−	+	−	−
5-Keto-D-gluconate	+	−	−	+	−	−	+	+
L-Lactate-alkalinization	+	+	+	+	−	+	−	+
Alpha-glucosidase	+	−	−	−	+	−	+	−
Alpha-galactosidase	+	−	+	+	−	+	+	−
Phosphatase	+	+	+	−	+	+	+	−
Glycine arylamidase	−	−	+	−	+	+	−	−
Ornithine decarboxylase	+	+	−	−	−	−	+	+
Lysine-decarboxylase	+	−	+	−	−	+	+	+
L-Histidine assimilation	+	−	+	−	−	+	+	−
Coumarate	−	+	−	−	+	−	+	+
Beta-glucoronidase	+	−	−	−	−	−	+	−
O/129 resistance	+	+	+	+	−	+	+	+
Glu-Gly-Arg-arylamidase	−	−	−	−	+	−	+	+
L-Malate assimilation	+	−	−	−	−	−	+	−
Ellman	−	+	−	+	−	−	−	−
L-Lactate assimilation	+	−	−	−	−	−	+	−

Table 7. The biochemical characteristics of gram-positive nonspore-forming bacterial isolates based on VITEK-2 bacterial identification system.

Test	Isolates		
	Sn5	Sn12	Sn15
D-Amygdalin	+	+	+
Phosphatidylinositol phospholipase C	+	+	+
D-Xylose	+	−	+
Arginine dihydrolase 1	+	+	+
Beta-galactosidase	−	−	−
Alpha-galactosidase	−	−	−
Ala-Phe-Pro arylamidase	+	−	+
Cyclodextrin	+	−	+
L-Aspartate arylamidase	+	+	+
Beta-galactopyranoside	+	−	+
Alpha-mannosidase	+	−	+
Phosphatase	−	+	−
Leucine arylamidase	+	−	+
L-Proline arylamidase	+	−	+
Beta-glucuronidase	+	−	+
Alpha-galactosidase	−	−	−
L-Pyrrolidonyl-arylamidase	+	−	+
Beta-glucuronidase	+	−	+
Alanine arylamidase	+	−	+
Tyrosine arylamidase	+	−	+
D-Sorbitol	+	+	+
Urease	−	−	−
Polymyxin B resistance	+	−	+
D-Galactose	+	+	+
D-Ribose	+	+	+
L-Lactate alkalinization	−	+	−
Lactose	+	−	+
N-Acetyl-D-glucosamine	−	−	−
D-Maltose	+	+	+
Bacitracin resistance	−	+	−
Novobiocin resistance	+	+	+
Growth in 6.5% NaCl	−	+	−
D-Mannitol	+	+	+
D-Mannose	+	+	+
Methyl-B-D-glucopyranoside	+	+	+
Pullulan	−	−	−
D-Raffinose	+	−	+
O/129 resistance	+	+	+
Salicin	−	+	−
Saccharose/sucrose	+	+	+
D-Trehalose	+	+	+
Arginine dihydrolase 2	+	−	+
Optochin resistance	+	+	+

Table 8. The biochemical characteristics of *Bacillus* isolates based on VITEK-2 bacterial identification system.

Tests	Isolates			
	Sn7	Sn8	Sn9	Sn10
L-Lysine arylamidase	+	+	+	+
L-Malate assimilation	+	+	+	+
Leucine-arylamidase	+	+	+	+
Arginine GP	+	+	+	+
Erythritol assimilation	+	+	+	+
Glycerol assimilation	–	–	–	–
Tyrosine arylamidase	–	–	–	–
B-N-Acetyl-glucosaminidase	+	+	+	+
Arbutin assimilation	+	+	+	+
Amygdalin assimilation	+	+	+	+
D-Galactose assimilation	+	+	+	+
Gentiobiose assimilation	+	+	+	+
D-Glucose assimilation	–	–	–	–
Lactose assimilation	+	+	+	+
Methyl-A-D-	+	+	+	+
D-Cellobiose assimilation	+	+	+	+
Gamma-glutamyl-transferase	–	–	–	–
D-maltose assimilation	+	+	+	+
D-Raffinose assimilation	+	+	+	+
PNP-N-acetyl-BD-galactosaminidase 1	+	+	+	–
D-Mannose assimilation	+	+	+	–
D-Melibiose assimilation	+	+	+	–
D-Melezitose assimilation	–	–	–	–
L-Sorbose assimilation	+	+	+	+
L-Rhamnose assimilation	+	+	+	+
Xylitol assimilation	+	+	+	+
D-Sorbitol assimilation	–	–	–	–
Saccharose/sucrose assimilation	+	+	+	–
Urease	–	–	–	–
Alpha-galactosidase	+	+	+	+
D-Turanose assimilation	–	–	–	–
D-Trehalose assimilation	+	+	+	+
Nitrate assimilation	–	–	–	–
L-Arabinose assimilation	+	+	+	+
D-Galacturonate assimilation	+	+	+	+
Esculin hydrolysis	+	+	+	+
L-Glutamate assimilation	–	–	–	–
D-Xylose assimilation	+	+	+	+
DL-Lactate assimilation	+	+	+	+
Acetate assimilation	–	–	–	–
Citrate (Sodium) assimilation	+	+	+	+
Glucoronate assimilation	+	+	+	+
L-Proline assimilation	+	+	+	+
2-Keto-D-gluconate assimilation	+	+	+	+
N-Acetyl-glucosamine assimilation	+	+	+	+
D-Gluconate assimilation	+	+	+	+

Table 9. Identification of the bacterial isolates from *S. nonagrioides* larvae based on the BLAST searches using 16S rRNA gene sequences.

Isolates	Suggested identification from GenBank	Query coverage (%)	16S rRNA similarity (%)	GenBank accession numbers
	Achromobacter insolitus LMG 6003	94	99	NR_025685.1
Sn1	*Achromobacter spanius* LMG 5911	94	99	NR_025686.1
	Achromobacter denitrificans DSM 30026	94	99	NR_042021.1
	Morganella morganii subsp. *morganii* KT	98	99	NR_102517.1
Sn2	*Morganella morganii* subsp. *sibonii* DSM 14850	93	99	NR_043751.1
	Morganella morganii M11	98	99	NR_028938.1
	Klebsiella variicola F2R9	95	99	NR_025635.1
Sn3	*Klebsiella variicola* At-22	98	99	NR_074729.1
	Klebsiella pneumoniae DSM 30104	98	99	NR_036794.1
	Citrobacter freundii DSM 30039	98	99	NR_028894.1
Sn4	*Citrobacter murliniae* CDC 2970–59	99	99	NR_028688.1
	Citrobacter braakii 167	99	99	NR_028687.1
	Arthrobacter mysorens DSM 12798	99	98	NR_025613.1
Sn5	*Arthrobacter uratoxydans* DSM 20647	99	98	NR_026238.1
	Arthrobacter protophormiae DSM 20168	99	98	NR_026195.1
	Chryseobacterium jejuense JS17-8	99	98	NR_044300.1
Sn6	*Chryseobacterium aquifrigidense* CW9	99	98	NR_044334.1
	Chryseobacterium indologenes LMG 8337	99	98	NR_042507.1
	Bacillus cereus ATCC 14579	97	99	NR_074540.1
Sn7	*Bacillus thuringiensis* IAM 12077	97	99	NR_043403.1
	Bacillus anthracis ATCC 14578	97	99	NR_041248.1
	Bacillus safensis FO-036b	92	99	NR_041794.1
Sn8	*Bacillus pumilus* ATCC 7061	92	99	NR_043242.1
	Bacillus pumilus SAFR-032	96	99	NR_074977.1
	Bacillus cereus ATCC 14579	99	99	NR_074540.1
Sn9	*Bacillus anthracis* Ames	99	99	NR_041248.1
	Bacillus thuringiensis IAM 12077	97	99	NR_074453.1
	Bacillus cereus ATCC 14579	99	99	NR_074540.1
Sn10	*Bacillus thuringiensis* IAM 12077	97	99	NR_043403.1
	Bacillus thuringiensis Bt407	99	99	NR_102506.1
	Klebsiella pneumoniae subsp. *pneumoniae* HS11286	98	99	NR_103936.1
Sn11	*Klebsiella variicola* F2R9	95	99	NR_025635.1
	Klebsiella pneumoniae DSM 30104	98	99	NR_036794.1
	Staphylococcus sciuri subsp. *sciuri* DSM 20345	100	100	NR_025520.1
Sn12	*Staphylococcus sciuri* subsp. *carnaticus* GTC 1227	100	99	NR_041327.1
	Staphylococcus sciuri subsp. *rodentium* GTC 844	100	99	NR_041328.1
	Citrobacter gillenii CDC 4693-86	99	98	NR_041697.1
Sn13	*Enterobacter kobei* CIP 105566	99	98	NR_028993.1
	Citrobacter freundii DSM 30039	99	98	NR_028894.1
	Serratia nematodiphila DZ0503SBS1	99	99	NR_044385.1
Sn14	*Serratia marcescens* subsp. *sakuensis* KRED	99	99	NR_036886.1
	Serratia marcescens WW4	99	99	NR_102509.1
	Microbacterium arborescens DSM 20754	94	99	NR_029265.1
Sn15	*Microbacterium imperiale* DSM 20530	94	99	NR_026161.1
	Microbacterium ginsengisoli Gsoil 259	95	98	NR_041516.1

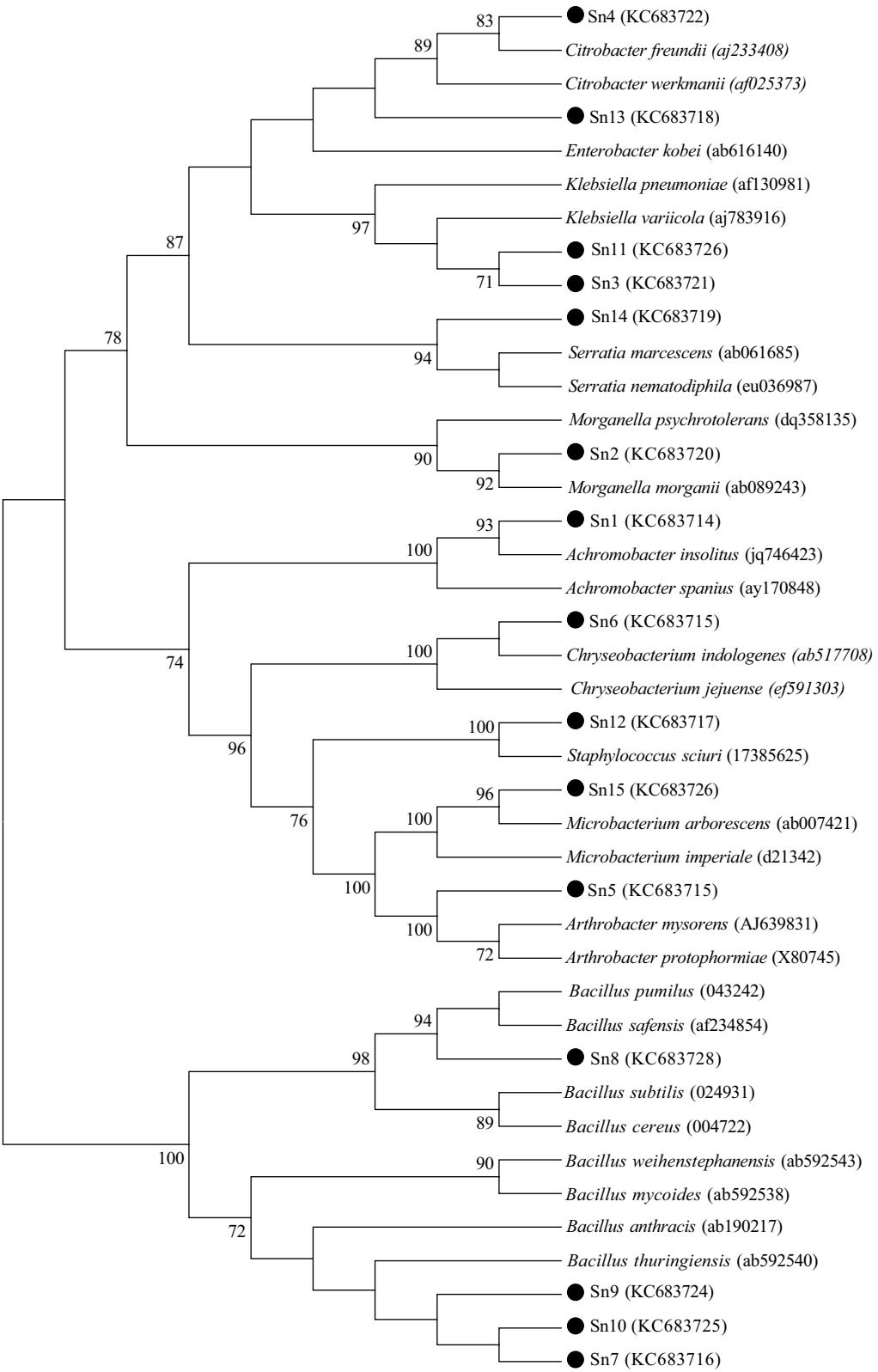

Figure 1. Neighbor-joining tree of 15 new isolates from *S. nonagrioides* and their 41 closely related species based on the partial sequence of 16S rRNA gene. *S. nonagrioides* isolates are indicated with a black dot. Bootstrap values shown next to nodes are based on 1000 replicates. Bootstrap values of C ≥ 70% are labeled.

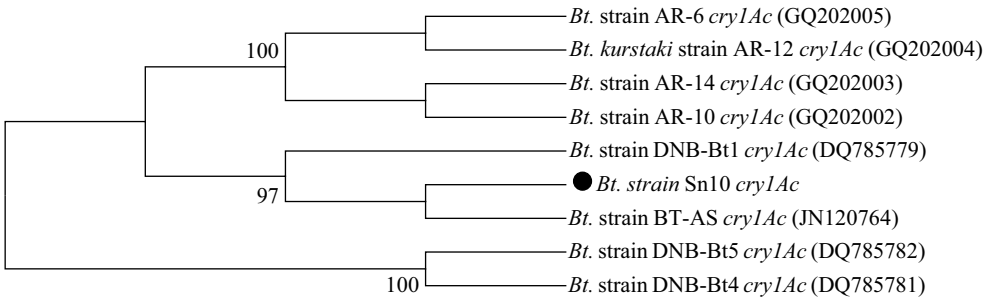

Figure 2. Neighbor-joining tree of Sn10 *cry*1Ac gene and closely related species based on the partial sequence of *cry*1Ac gene. Sn10 *cry*1Ac is indicated with a black dot. Bootstrap values shown next to nodes are based on 1000 replicates (*Bt.*: *Bacillus thuringiensis*).

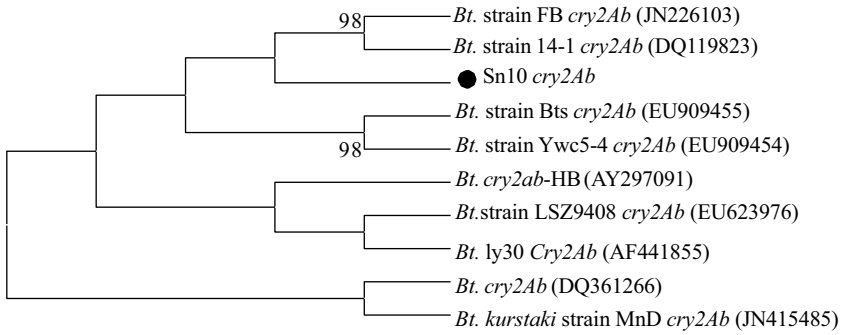

Figure 3. Neighbor-joining tree of Sn10 *cry*2Ab gene and closely related species based on the partial sequence of *cry*2Ab gene. Sn10 *cry*2Ab is indicated with a black dot. Bootstrap values shown next to nodes are based on 1000 replicates (*Bt.*: *Bacillus thuringiensis*).

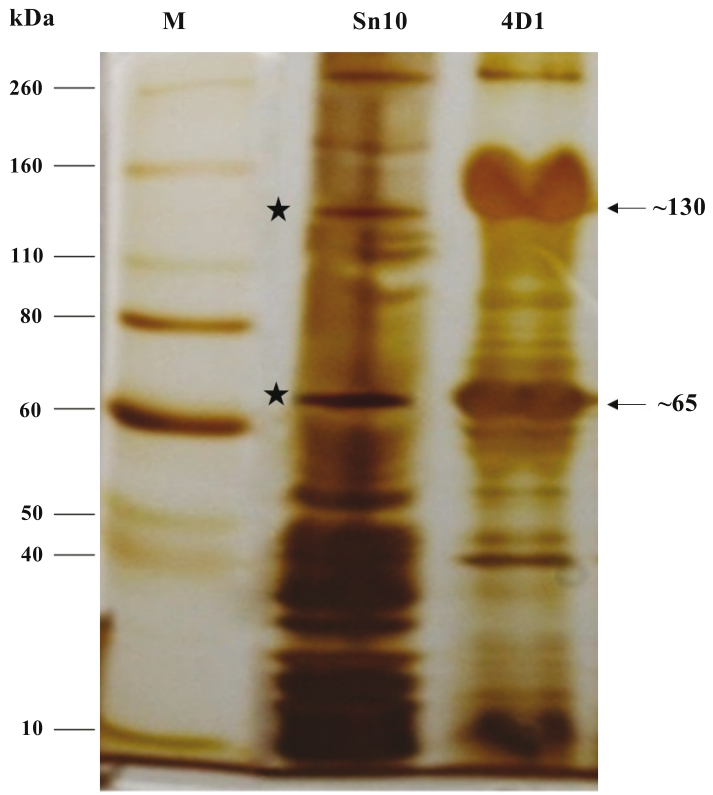

Figure 4. SDS-PAGE of crystal proteins of *B. thuringiensis* Sn10. M: Molecular marker, 4D1: *B. thuringiensis* reference strain. Stars indicate cry1 protein of ~130 kDa and cry2 protein of ~65 kDa.

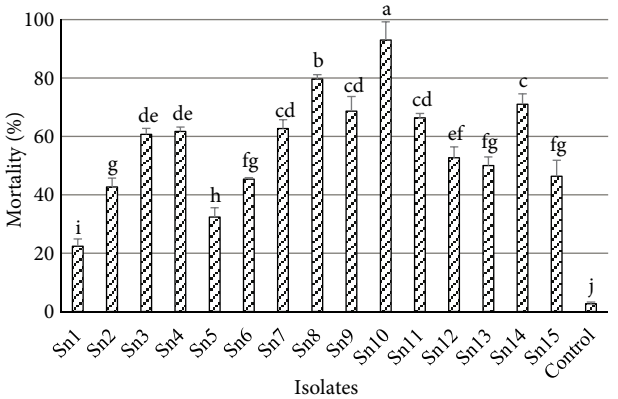

Figure 5. Pathogenicity of the bacterial isolates of *S. nonagrioides* against third-instar larvae of *S. nonagrioides*. Mortality data were corrected with Abbott's formula (Abbott, 1925). Different lowercase letters represent statistically significant differences between mortalities according to LSD multiple comparison test ($P < 0.05$). Bars show standard error.

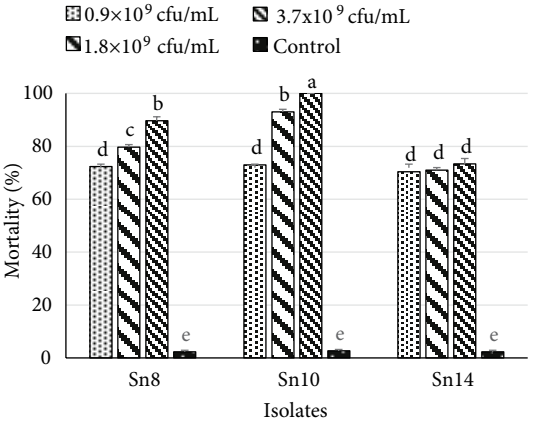

Figure 6. Dose-response experiments of Sn8, Sn10, and Sn14 isolates against third-instar larvae of *S. nonagrioides*. Mortality data were corrected with Abbott's formula (Abbott, 1925). Different lowercase letters represent statistically significant differences between mortalities according to LSD multiple comparison test ($P < 0.05$). Bars show standard error.

Table 10. LC_{50} and LC_{95} (cfu/mL) values of Sn8, Sn10, and Sn14 isolates against *S. nonagrioides* larvae.

Isolates	LC_{50} (FL, 95%)	Slope ± SE	LC_{95} (FL, 95%)	df	X^2
Sn8	0.258×10^9 (0.008×10^9 to 0.568×10^9)	1.34 ± 0.341	10.32×10^9	1	0.0523
Sn10	0.596×10^9 (0.38×10^9 to 0.74×10^9)	2.71 ± 0.102	1.88×10^9	1	0.0273
Sn14	0.0002×10^9 (0.00011×10^9 to 0.0029×10^9)	0.19 ± 23.5	$58,899,447.8 \times 10^9$	1	0.0022

FL: fiducial limit, SE: standard error, df: degree of freedom, X^2: chi square.

first time, this work isolated *Achromobacter insolitus* (Sn1) from an insect. Therefore, the current study is significant for the determination of bacterial flora in *S. nonagrioides*. A previous study about the isolation of bacteria from *S. nonagrioides* was performed by Esfandiari and Motamedi (2013). They isolated 11 bacteria from *S. nonagrioides*, including *Acinetobacter calcoaceticus*, *A. baumannii*, *A. radioresistens*, *A. lwoffii*, *Enterococcus casseliflavus*, *E. gergoviae*, *Cedecea lapagei*, *Kurthia gibsonii*, *Staphylococcus auricularis*, *Listeria ivanoii*, and *Erwinia herbicula*, based on morphology, spore formation, nutritional features, and physiological and biochemical characteristics. However, they did not use molecular methods for identification and did not perform a pathogenicity assay of bacterial flora against *S. nonagrioides*. In this study, we used molecular methods for identification in addition to conventional methods. We also tested the pathogenicity of bacterial flora members against *S. nonagrioides*. Bacterial flora was found to be different from previous studies. The reason for this difference is thought to be the geographic position and nutritional regime. While previous studies determined bacterial flora from dead larvae, we used live larvae for its determination. This may be the reason for the discrepancy between bacterial flora.

Achromobacter insolitus is a member of the family Alcaligenaceae, which was isolated from various human clinical samples. So far, it had not been isolated from insects. *Achromobacter insolitus* (Sn1) was isolated from an insect for the first time and did not have significant insecticidal effect (25%) against *S. nonagrioides*.

Morganella morganii is commonly found in the environment and in the intestinal tracts of humans, mammals, and reptiles as normal flora. Despite its wide distribution, it is an uncommon cause of community-acquired infection and is most often encountered in postoperative and other nosocomial settings. *M. morganii* has also been isolated from various insect species (Trasmonte et al., 2009; Gupta et al., 2012). However, Nishiwaki et al. (2007) isolated *Morganella morganii* from predatory larvae of the antlion species *Myrmeleon bore* and it had considerable insecticidal effect (100%) on the pest. We also isolated *M. morganii* from *S. nonagrioides* and it had 43% mortality.

Klebsiella spp. are closely associated with many insect species, and species belonging to this genus are generally not insect pathogens. In all probability, they play a role in the digestion process in the insect gut and in the physiological developments of larvae (Demir et al., 2002; Ademolu and Idowu, 2011). In this study, we also isolated 2 *Klebsiella pneumoniae* (Sn3 and Sn11) strains from *S. nonagrioides*. Both these isolates caused 66% mortality in the pest.

C. freundii is responsible for a number of significant opportunistic infections. It is known to be the cause of a number of nosocomial infections of the respiratory tract, urinary tract, blood, and many other normally sterile sites in patients (Whalen, 2007). This organism's ecological role includes its important role in the nitrogen cycle, and it can also accumulate uranium by building phosphate complexes (Puchenkova, 1996). *C. freundii* has been isolated from various insect species (Yuval et al., 2013). In the bioassays, *C. freundii* showed 63% mortality in *S. nonagrioides* larvae.

Arthrobacteria are coryneform bacteria. They are widely distributed in the soil. *Brevibacterium protophormia* was isolated from *Protophormia terraenovae* by Lysenko (1959), and reclassification of *Brevibacterium protophormiae* as *Arthrobacter protophormiae* was done by Stackebrandt et al. (1983). In the present work, this strain caused low mortality (32%) in *S. nonagrioides* larvae.

Chryseobacterium indologenes was isolated from various insects (Yabuuchi et al., 1983; Buresova et al., 2006). *C. indologenes* was first isolated from a clinical specimen and characterized by Yabuuchi et al. (1983). *C. indologenes* should be considered as a potential pathogen in newborns in the presence of invasive equipment or in treatment with long-term broad-spectrum antibiotics. *C. indologenes* (Sn6) had 45% mortality in *S. nonagrioides* larvae.

Bacterial pathogens used for insect control are spore-forming, rod-shaped bacteria in the genus *Bacillus.* This genus is the most studied group of bacteria in insect pathology (Boemare and Tailliez, 2010) and species are commonly recognized as definitive insect pathogens (Stahly et al., 2006; Masetti et al., 2008). Among the *Bacillus* species, *Bacillus thuringiensis* (Sn7, Sn9, and Sn10) has been isolated from several insect species belonging to a variety of different orders at various times (Omoya and Akinyosoye, 2011; Sevim et al., 2012). Spores and crystalline insecticidal proteins produced by *B. thuringiensis* have been used to control insect pests since the 1920s (Wei et al., 2003). Because of their specificity, these pesticides are regarded as environmentally friendly, with little or no effect on humans, wildlife, pollinators, and most other beneficial insects, and they are used in organic farming. Cry1Ac and Cry2Ab toxins were detected in Sn10 (*B. thuringiensis*). Traditionally, these toxins are effective against insects in Lepidoptera (Albernaz et al., 2013). Accordingly, Sn10 had 93% mortality in the pest. In addition, the insecticidal activity of Sn10 increased by 100% at a 2-fold concentration. However, Özkan Çakıcı et al. (2013) isolated and characterized a *Bacillus safensis* strain from *C. quadrimaculatus* for the first time; it showed 100% insecticidal effect on *C. quadrimaculatus*. We also found that *B. safensis* from *S. nonagrioides* is an effective pathogen that had 80% insecticidal activity on *S. nonagrioides* larvae.

So far, *Staphylococcus* species have been isolated from different insect species such as *Anastrepha ludens*, *Hylesia metabus*, *Hepialus gonggaensis*, *Toxoptera aurantii*, and *Thaumetopoea pityocampa* (Yu et al., 2008), although Bucher (1981) indicated that *Staphylococcus* species are rarely associated with insects. We also isolated *Staphylococcus sciuri* (Sn12) from *S. nonagrioides* larvae and found it to have 54% mortality in the pest.

Enterobacter spp., which naturally occur in large numbers in the intestines of mammals, birds, reptiles, and insects, are closely related to many insect species. Some studies have shown that species belong to this genus differ in pathogenicity value on insects (Demir et al., 2002; Sevim et al., 2010b; Ademolu and Idowu, 2011). These bacteria are not regarded as primary pathogens, but are generally recognized as nosocomial pathogens worldwide. *Enterobacter kobei* (Sn13) had approximately 50% insecticidal activity against the pest larvae. It most likely plays a role in the digestion processes in the insect gut and in the physiological development of *S. nonagrioides* larvae. These points may suggest that *Enterobacter*, a common symbiont in the gastrointestinal tracts of domestic animals, is possibly an insect pathogen. However, more detailed bioassay experiments are needed to prove this.

S. marcescens causes disease in a wide range of invertebrate and vertebrate hosts. This species is one of the most well-known bacterial pathogens of insects. Gokce et al. (2010) showed that *S. marcescens* Rb2 has 75% mortality in *Rhynchites bacchus* (L.) larvae. Bahar and Demirbağ (2007) found that *S. marcescens* Ol13 caused 65% mortality in *Oberia linearis* (L.) larvae. Our results are in compliance with these studies, and we also found that *Serratia marcescens* Sn14 produced 75% mortality in *S. nonagrioides* larvae.

M. arborescens was reclassified from *Flavobacterium arborescens* by Imai et al. (1984). Strains of the genus *Microbacterium* are widespread and can be isolated from different sources (Collins and Bradbury, 1992). *M. arborescens* SE14 was isolated from *Spodoptera exigua* by Ping et al. (2007). We also isolated *M. arborescens*, and it

showed 46% mortality in *S. nonagrioides* larvae.

In conclusion, we isolated and characterized 15 bacteria from *S. nonagrioides* larvae and tested their effectiveness against it. Some of the isolates appeared to be significant candidates for the biological control of this pest. *B. thuringiensis* (Sn10) and *B. safensis* (Sn8) are especially the most promising isolates for the microbial control of *S. nonagrioides*. Findings from this study showed that further studies should include field efficacy of the isolates Sn10 and Sn8 and an investigation of the predisposition of those isolates in terms of mass production.

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