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A study on the characterization and pathogenicity of bacteria from *Lymantria dispar* L. (Lepidoptera: Lymantriidae)

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Abstract: Gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae), is one of the most serious pests affecting fruit, forest, industrial, and foliage plants worldwide. The aim of this study was to isolate and characterize pathogenic bacteria from this pest and determine their pathogenicity. *L. dispar* larvae were collected from various populations in the forest areas of the Black Sea region of Turkey in May and June of 2008 and 2009, and 9 culturable bacterial isolates were obtained from these larvae. Morphological and physiological characteristics of bacterial isolates were determined by conventional and routine techniques, and biochemical properties and metabolic enzyme profiles were revealed using API20E and Phoenix 1000A panel test systems. Additionally, a 16S rRNA gene sequence analysis was performed to identify the isolates at the molecular level and to query against the NCBI genetic database. Consequently, 7 of the bacterial isolates were determined and characterized at the species level while the rest were characterized at the genus level. From the results of all tests, the isolates were identified as *Pantotea agglomerans* (Lyd1), *Klebsiella* sp. (Lyd2), *Acinetobacter calcoaceticus* (Lyd3), *Serratia marcescens* (Lyd4), *Erwinia* sp. (Lyd5), *Bacillus thuringiensis* (Lyd6), *B. thuringiensis* (Lyd7), *B. thuringiensis* (Lyd8), and *B. thuringiensis* (Lyd9). Of all bacterial isolates tested for insecticidal activity, 2 of the *B. thuringiensis* isolates (Lyd6 and Lyd9) were found to cause the highest insecticidal activity (90%) within 10 days of application. Furthermore, 100% insecticidal activity was observed with a crystal-spore mixture of the same isolates against *Lymantria dispar* larvae within 10 days.

Key words: *Lymantria dispar*, culturable bacterial flora, insecticidal activity, *Bacillus thuringiensis*

Introduction

The gypsy moth, *Lymantria dispar*, is one of the most important and dangerous herbivorous insects. It is a major foliage pest of deciduous trees throughout the northern hemisphere and in many other countries around the world (1). This pest is highly polyphagous and damages more than 500 hosts belonging to different plant families. Furthermore, it is particularly harmful for hazelnut trees and other plants including agricultural, forestry, and ornamental plants in the Black Sea region of Turkey (2). Gypsy moths cause the most damage to trees and shrubs during their larval or caterpillar stage. Large infestations can completely defoliate large mature trees. The larvae move to the leaves of trees and begin to eat, mostly at night. During daylight, the larvae generally seek shade from the sun; however, feeding can occur in the daytime in heavy infestations. The trees may become completely leafless in some outbreaks.

Some chemical substances (diflubenzuron, dichlorvos, and disparlure) have been utilized to control this pest in Turkey and other countries (2,3). However, recent concerns about the hazardous effects of chemical pesticides on the environment have encouraged scientists to find safer, more effective control agents. Biological control agents such as

predators, parasitoids, and/or natural pathogens can be used as an alternative or a supplement to chemical control agents (4-7). Effective biological control agents are host-specific and persist in the environment, thus obviating the need for repeat applications. The use of microbial control agents against *L. dispar* has been studied previously. Microbial insecticides based on entomopathogenic bacteria (8), viruses (9), and fungi (10,11) have been assessed as possible microbial control agents for *L. dispar*. Despite this research, the effectiveness of the pest on a great number of plants remains all over the world.

Studies of symbiotic bacteria in insect species facilitate the development of new approaches to microbial control. Symbiotic bacteria are ubiquitously located in animal guts, with symbioses ranging from pathogenic to mutualistic and facultative to obligate (12). In the last 50 years, numerous bacteria, such as *Bacillus sphaericus* and *B. thuringiensis*, have been isolated, classified, and demonstrated in the laboratory to be pathogens to various insects (13-17). Approximately 60 pathogenic species of bacteria and their products have been developed as pesticides worldwide, and various bacterial insect pathogens are successfully being used in the microbial control of insects (18,19).

Several studies have been performed to determine the different entomopathogens in *L. dispar* (20,21), but studies of the culturable bacterial flora of *L. dispar* and their insecticidal effects on the pest are lacking. Therefore, in order to find a more effective and safe biocontrol agent against gypsy moth, we investigated the culturable bacterial flora of *L. dispar* collected from the Black Sea region in Turkey by utilizing current morphological, biochemical, and molecular techniques. In this study, we isolated 9 culturable bacterial isolates, characterized them in detail, and tested their insecticidal activities against *L. dispar* larvae.

Material and methods

Collection of gypsy moth larvae

Larvae of the gypsy moth at different instars were used for the isolation of bacteria. Larvae were collected on a daily basis from different hazelnut fields in the vicinity of Trabzon in June 2008. They were placed individually into plastic boxes (20 mm) with perforated covers to permit airflow, along with some hazelnut leaves. The boxes were transported to the microbiology laboratory of the Karadeniz Technical University Department of Biology, in Trabzon, Turkey. Larvae were fed *Corylus* leaf at room temperature with a 12:12 photoperiod until bacterial isolation.

Isolation and purification of culturable bacteria from gypsy moth larvae

After macroscopic examination, healthy and dead larvae were distinguished. Ten living larvae were individually placed into 70% ethanol, gently swirled for 2 min, and washed twice in sterile distilled water. Using standard aseptic techniques, surface-sterilized larvae were individually dissected in sterile water. The insect bodies were homogenized with nutrient broth media in test tubes containing 3 mL of nutrient broth (Difco) using a glass tissue grinder. Suspensions were diluted from 10^{-5} to 10^{-8}, and a 50-µL suspension was plated on nutrient agar medium in petri dishes for bacterial isolation (18). Plates were incubated at 30 °C for 2-3 days. At the end of the incubation period, discrete bacterial colonies on nutrient agar were aseptically removed using an inoculation loop and incubated aerobically for 24-48 h. Individual colonies were isolated, subcultured twice to ensure purity, and then stored in 15% sterilized glycerol at −80 °C for further study.

Identification of bacterial isolates based on conventional tests

Bacterial cultures were identified by their morphology, spore formation, and nutritional features; by their physiological, biochemical, and molecular characteristics; and by the VITEK 32 bacterial identification system.

The morphological, physiological, and biochemical features of the bacterial isolates were determined according to *Bergey's Manual of Systematic Bacteriology*, Volumes 1 and 2 (22,23), and the *Manual of Techniques in Insect Pathology* (18). Gram staining was performed according to the procedure described by Claus (24). Endospore staining was performed according to the method of Prescott et al. (25), and capsule staining was performed by negative staining. The motility of isolates was determined using a semisolid medium (26). Analytical profile

indexes (APIs) from kit API 20E, kit API 50CH, and VITEK systems (bioMerieux, Hazelwood, MO, USA) were used for identification of the physiological and biochemical properties of the bacterial isolates.

API panel test systems

Substrate utilization tests were performed using API 20E and API 50CH panel systems. API test strips were handled according to the manufacturer's instructions (bioMerieux, Marcy l'Etoile, France) with a few small modifications. Stock cultures were streaked onto nutrient agar to obtain single colonies for each bacterial isolate. Bacterial colonies of each isolate were diluted in 0.85% NaCl solution. The amount of bacteria was adjusted to 1 McFarland, and 200 mL of this solution was transferred into each well of the panels. In order to prevent contamination from the air, the wells were filled with mineral oil, and then the panels were incubated for 18-24 h at 30 °C. The results of the tests were evaluated using the computer program IdBact v. 1.1, G. Kronvall, with the matrix for API from bioMerieux, France.

Isolate identification by VITEK 32

The stock culture strains were subcultured onto tryptic soy agar plates to check their purity. A standard bacterial suspension was prepared in 1.8 mL of 0.45% saline using the VITEK colorimeter for each isolate. The time interval between suspension preparation and card filling was less than 30 min to avoid changes in turbidity. After the preparation of the suspension it was inoculated into the VITEK GNI (for gram-negative bacteria), GPI (for gram-positive bacteria), and CAP (for rod-shaped bacillus bacteria) cards; incubated at 35 °C in the reader incubator module for 18 h; and automatically read hourly by the optical scanner using the bioLiaison software according to the manufacturer's recommendations. Since the VITEK system requires the oxidase (gram-negative) and catalase (gram-positive) test results for identification, we also determined the features of the oxidase and catalase tests (27).

16S rRNA gene sequencing

In order to extract DNA from the bacterial isolates, bacterial colonies were inoculated into nutrient broth and incubated at 30 °C overnight (approximately 18 h) with shaking at 200 rpm in a dry air incubator. At the end of the growth period, overnight cultures were transferred to 1.5-mL microcentrifuge tubes and centrifuged at 13,000 × *g* for 2 min to pellet the cells. This step was repeated twice to collect all bacterial cells. After that, genomic DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega, Mannheim, Germany) according to the manufacturer's recommendations.

Universal primers, UNI16S-L; 5'-ATTCTAGAGTTTGATCATGGCTCA-3' and UNI16S-R; 5'-ATGGTACCGTGTGACGGGCGGTGTGTA-3', were used as the forward and reverse, respectively, in the polymerase chain reaction (PCR) (28). Amplifications were performed in a total volume of 50 µL, which included 1.5 µL of 10 mM dNTP mix, 1.5 µL of 10 pmol each of the opposing amplification primers, 1 µL of 5 U/µL Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 3 µL of $MgCl_2$, 5 µL of Taq DNA polymerase reaction buffer, and 2 µL of genomic DNA; the final volume was completed with the addition of dH_2O. PCR amplifications were performed with the Bio-Rad Thermal Cycler (Hercules, CA, USA). The reaction conditions contained an initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation of 1 min at 94 °C, 1 min at 53.9 °C, and 1 min at 72 °C, with a final extension at 72 °C for 5 min. Amplified 16S rRNA gene products were separated by electrophoresis on 1% agarose gel and excised from the gel using the NucleoSpin Extract II Kit (Clontech, Mountain View, CA, USA). Gel-purified PCR products of the 16S rRNA gene fragments from 5 isolates were cloned directly into the pGEM-T vector cloning system and transformed into an *Escherichia coli* DH5α strain. After selection of transformed colonies, plasmid isolation was performed and plasmid DNA samples were digested by restriction enzymes in order to confirm whether the gene had been cloned into the vector or not. Plasmid DNA samples that were the right clone were sent to Macrogen, Korea, for sequencing. The obtained data sequences were then submitted to GenBank for accession numbers and were compared with known 16S rRNA gene sequences in the GenBank database using the BLAST search algorithm. Finally, the taxonomic identification of bacterial isolates was determined by comparing the results obtained by conventional tests and those from API, VITEK 32, and 16S rRNA gene analysis data.

Insecticidal effects of bacterial isolates

Bioassays were performed to determine the insecticidal effects of the bacterial isolates on the third instar larvae of *L. dispar*. First, all of the bacterial isolates were tested as overnight cultures after removing the growth media. *B. thuringiensis* isolates (Lyd6, Lyd7, Lyd8, and Lyd9) were then applied using a purified crystal and spore mixture. Healthy larvae were used for the insecticidal activity tests of bacterial isolates. Isolates were incubated for 18 h at 30 °C in nutrient broth. After incubation, the density of the cells was set at 1.89 at OD_{600}, and 5 mL of culture was centrifuged at 3000 rpm for 10 min. The pellet was then resuspended in 5 mL of sterilized PBS and used for bioassays (29). Isolation of crystal and spore mixtures from *B. thuringiensis* isolates was performed according to the method of Aptosoglou et al. (30), with some modifications. After incubation for 5 days at 30 °C on nutrient agar plates for sporulation, the bacteria were collected from the agar surface. The mixture of spores and crystals was suspended in 0.5 M NaCl and centrifuged at 13,800 × *g* for 5 min. The pellet was resuspended with distilled water at a concentration of 100 µg/mL. The bacterial suspension and the crystal-spore mixtures were used to contaminate fresh leaves of *Corylus* sp., which were used as food in the bioassays, and these were placed into individual sterile plastic containers (80 mm in diameter). For the control group, sterile water was applied to leaves of *Corylus* sp. Ten third instar larvae, 4 h starved, were then placed on the contaminated leaves in containers; the containers were kept at 26 ± 2 °C and 60% relative humidity with a 12:12 photoperiod (31). The mortalities of the larvae were recorded every 24 h, with all dead larvae removed from the containers until 10 days after inoculation of the isolates. The data were evaluated using Abbott's formula (32).

Results

A total of 9 culturable isolates were finally selected and characterized according to their morphology, spore formation, nutritional features, physiological and biochemical characteristics; the results of the API and the VITEK 32 system; and analysis of the 16S rRNA gene sequence (Tables 1-3). All of the bacterial isolates were obtained from healthy larvae.

The color of colonies on the agar plates was cream for 6 isolates (Lyd2, Lyd3, Lyd6, Lyd7, Lyd8, and Lyd9), yellowish for 2 isolates (Lyd1 and Lyd5), and red for 1 isolate (Lyd4). The colony morphology was round for 5 isolates (Lyd1, Lyd2, Lyd3, Lyd4, and Lyd5) and rough for 4 isolates (Lyd6, Lyd7, Lyd8, and Lyd9).

The morphology of the bacterial isolates was bacillus (Lyd1, Lyd3, Lyd4, Lyd6, Lyd7, Lyd8, and Lyd9), coccobacillus (Lyd2), and coccus (Lyd5). Five isolates (Lyd1, Lyd2, Lyd3, Lyd4, and Lyd5) were gram-negative and the others (Lyd6, Lyd7, Lyd8, and Lyd9) were gram-positive. All gram-positive isolates (Lyd6, Lyd7, Lyd8, and Lyd9) were spore-forming bacteria. Motility was observed in 6 isolates (Lyd4, Lyd6, Lyd7, Lyd8, and Lyd9). All isolates caused turbidity in the nutrient broth medium (Table 1).

Table 1. The morphological characteristics of bacterial isolates.

Tests \ Isolates	Lyd1	Lyd2	Lyd3	Lyd4	Lyd5	Lyd6	Lyd7	Lyd8	Lyd9
Color of colonies	Yellowish	Cream	Cream	Red	Yellowish	Cream	Cream	Cream	Cream
Shape of colonies	Round	Round	Round	Round	Round	Rough	Rough	Rough	Rough
Shape of bacteria	Bacillus	Coccobacillus	Bacillus	Bacillus	Coccus	Bacillus	Bacillus	Bacillus	Bacillus
Gram stain	–	–	–	–	–	+	+	+	+
Spore stain	–	–	–	–	–	+	+	+	+
Motility	–	–	–	+	–	+	+	+	+

+/–: positive and negative results for corresponding morphology.

The physiological properties of the isolates are reported in Table 2. While no isolates grew at pH 4 and 12.5, all isolates grew at pH 5.5 and 12. The optimal growth was seen at pH 7 for all isolates. For all isolates grown at 4% NaCl, growth decreased along with increasing concentration. Temperature tests revealed that no isolate grew at temperatures of 15 °C and lower; all isolates grew at 30 °C and 37 °C; and only Lyd6, Lyd7, Lyd8, and Lyd9 grew at 40 °C.

Some biochemical characteristics were also investigated, and the results are reported in Table 3. Indole and gelatin tests were negative, but catalase was positive for all the isolates. Nitrate reduction and glucose fermentation tests were negative only for Lyd3. Rhamnose fermentation was positive only for Lyd5, while the citrate test was positive for Lyd3 and Lyd4. Other tests also varied among isolates, as shown in Table 3. For more detailed identification based on the biochemical properties of the bacterial isolates, the API panel and VITEK 32 identification systems were used.

The 16S rRNA genes of isolates were amplified by PCR and sequenced for further characterization. Approximately 1400-bp fragments of the 16S rRNA gene region of all isolates were sequenced. The results of this gene sequence were used to query the NCBI genetic database to evaluate their relationships with related bacterial isolates.

Consequently, based on their morphological, physiological, and molecular characteristics, while 7 isolates (Lyd1, Lyd3, Lyd4, Lyd6, Lyd7, Lyd8, and Lyd9) of the 9 culturable isolates from *Lymantria dispar* were identified at the species level, 2 isolates (Lyd2 and Lyd5) could be identified only at the genus level. According to all assessments, the culturable bacterial flora of *L. dispar* were identified as *Pantotea agglomerans* (Lyd1), *Klebsiella* sp. (Lyd2), *Acinetobacter calcoaceticus* (Lyd3), *Serratia marcescens* (Lyd4), *Erwinia* sp. (Lyd5), and *Bacillus thuringiensis* (Lyd6, Lyd7, Lyd8, and Lyd9) (Table 4).

Table 2. The physiological characteristics of bacterial isolates.

Tests \ Isolates	Lyd1	Lyd2	Lyd3	Lyd4	Lyd5	Lyd6	Lyd7	Lyd8	Lyd9
pH 4	–	–	–	–	–	–	–	–	–
pH 5	+	–	+	–	+	–	–	–	–
pH 5.5	+	+	+	+	+	+	+	+	+
pH 12	+	+	+	+	+	+	+	+	+
pH 12.5	–	–	–	–	–	–	–	–	–
4% NaCl	+	+	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+*	+	+
7% NaCl	+	+	–	+	+	+*	–	–	+*
8% NaCl	+	–	–	+	+	–	–	–	–
9% NaCl	+*	+*	–	+*	+*	–	–	–	–
Growth at 15 °C	–	–	–	–	–	–	–	–	–
Growth at 30 °C	+	+	+	+	+	+	+	+	+
Growth at 37 °C	+	+	+	+	+	+	+	+	+
Growth at 40 °C	–	–	–	–	–	+	+	+	+

*: growth after 72 min.

Table 3. The biochemical characteristics of bacterial isolates.

Tests \ Isolates	Lyd1	Lyd2	Lyd3	Lyd4	Lyd5	Lyd6	Lyd7	Lyd8	Lyd9
Urease	−	−	−	+	+	−	+	−	−
Methyl red tests	−	+	−	−	+	+	+	+	+
Voges-Proskauer	+	−	−	+	−	+	+	−	+
Indole	−	−	−	−	−	−	−	−	−
Nitrate reduction	+	+	−	+	+	+	+	+	+
Citrate	−	−	+	+	−	−	−	−	−
Catalase	+	+	+	+	+	+	+	+	+
Gelatin	−	−	−	−	−	−	−	−	−
Mannitol	+	+	−	+	+	−	−	−	−
Rhamnose	−	−	−	−	+	−	−	−	−
MCA	+	+	+	+	+	−	−	−	−
Starch	−	−	−	−	−	+	+	+	+
KIA	A/A	A/B	?	A/A	A/A	A/A	A/B	A/B	A/B
Glucose	+	+	−	+	+	+	+	+	+
Arabinose	+	+	−	−	+	W	−	−	−
Lactose	−	−	−	−	+	W	−	−	W
Xylose	−	−	−	+	+	−	+	−	−

MCA: MacConkey agar, KIA: Kligler iron agar, W: weak, A/A: acid/acid, A/B: acid/base;
+/−: positive and negative results for corresponding tests.

Table 4. Taxonomic identification of bacterial isolates.

Isolate codes	Most likely identical taxonomic group and species	16S rRNA similarity (%)
Lyd1	*Pantotea agglomerans*	99
Lyd2	*Klebsiella* sp.	99
Lyd3	*Acinetobacter calcoaceticus*	99
Lyd4	*Serratia marcescens*	99
Lyd5	*Erwinia* sp.	99
Lyd6	*Bacillus thuringiensis*	99
Lyd7	*Bacillus thuringiensis*	99
Lyd8	*Bacillus thuringiensis*	99
Lyd9	*Bacillus thuringiensis*	99

In addition, we tested the insecticidal activity of the bacterial isolates on *Lymantria dispar* larvae. The insecticidal activity of isolates at 1.8×10^9 bacteria/mL doses within 10 days of application to *L. dispar* larvae were 40% for Lyd1, Lyd2, Lyd7, and Lyd8; 60% for Lyd3, Lyd4, and Lyd5; and 90% for Lyd6 and Lyd9. Furthermore, with the crystal protein-spore mixture of *B. thuringiensis* isolates, the highest insecticidal effect (100%) was detected for Lyd6 and Lyd9 (Figure).

Discussion

There is currently an increasing interest in finding more effective and safer biological control agents for hazardous insects. One of the best ways is to utilize the entomopathogens of harmful insects for biological control purposes. To date, this is the first study performed to determine culturable bacterial isolates and test the insecticidal effect of these isolates against *Lymantria dispar* larvae. Based on colony morphology among total bacterial isolates, 9 different isolates (Lyd1-Lyd9) were determined and characterized based on morphological, nutritional, physiological, and biochemical characteristics; API and the VITEK 32 system; and 16S rRNA gene sequencing (Tables 1-3). The phenotypic features were compared with *Bergey's Manual of Systematic Bacteriology*. Based on the results of all tests, which were in alignment with each other, the culturable bacterial flora of *L. dispar* consisted of *Pantotea agglomerans* (Lyd1), *Klebsiella* sp. (Lyd2), *Acinetobacter calcoaceticus* (Lyd3), *Serratia marcescens* (Lyd4), *Erwinia* sp. (Lyd5), *Bacillus thuringiensis* (Lyd6), *B. thuringiensis* (Lyd7), *B. thuringiensis* (Lyd8), and *B. thuringiensis* (Lyd9) (Table 4).

The bacterial species isolated in this study have been previously isolated from other insect species (14,15,17,19,33-37).

In this study, 4 *B. thuringiensis* strains (Lyd6, Lyd7, Lyd8, and Lyd9) were isolated from *L. dispar* (Table 4). *B. thuringiensis* has also been isolated from several other insects, including *Anoplus roboris* (14), *Agelastica alni* (38), *Melolontha melolontha* (39), and *Thaumetopoea pityocampa* (36,40). In addition, *B. thuringiensis* and some other species of the *Bacillus* genus, such as *B. cereus* (41), *B. subtilis* (42), *B. popilliae* (43), *B. circulans* (14), *B. polymyxa* (14), and *B. megaterium* (15), have been isolated from different insects. This demonstrates that *Bacillus* species are very common in insect populations.

The insecticidal activities of *Acinetobacter calcoaceticus*, *Serratia marcescens*, and *Erwinia* sp.

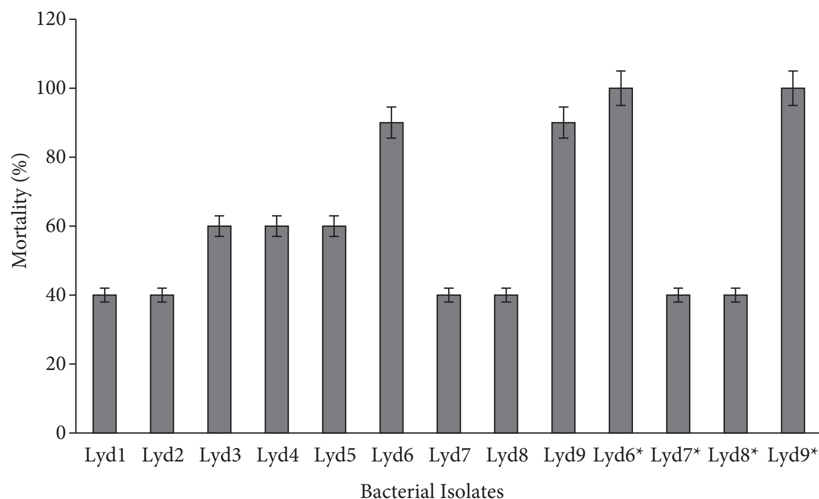

Figure. Mortality of the third instar larvae of *Lymantria dispar* 10 days after application of bacterial isolates and crystal protein-spore mixture. Mortality data corrected according to Abbott's formula; *: crystal-spore mixture.

in the *L. dispar* larvae were 60% within 10 days. In a previous study, in which *A. calcoaceticus* was isolated from *Agrotis segetum*, the isolate had a 20% insecticidal activity in the third instar larvae of *A. segetum* (17). The results of both Sevim et al. (17) and the current study indicate that *A. calcoaceticus* is a symbiotic and pathogenic bacteria for insect species, but it has different levels of virulence for different insects. *Serratia* species have also often been associated with insect diseases. This genus contains insect pathogenic strains that are usually considered opportunistic or facultative pathogens, as they are often virulent to insects when they are present in the digestive tract but are lethal upon entering the insect hemocoel following injury or stress (43). *S. marcescens* causes disease in plants and in a wide range of invertebrate and vertebrate hosts, and it has been isolated and used for the biocontrol of other pests (13,16,33,34). Sezen and Demirbag (13) reported that *S. marcescens* isolated from *Balaninus nucum* L. (Col.: Curculionidae) caused 100% mortality in larvae of *B. nucum* within 3 days. Bahar and Demirbağ (34) found that *S. marcescens* Ol13 (isolated from *Oberea linearis*) caused 65% mortality in the larvae of *O. linearis* within 10 days. Gokce et al. (16) reported that *S. marcescens* Rb2 isolated from *Rhynchites bacchus* produced 75% mortality in the larvae of *R. bacchus* within 10 days. Our results correspond with these studies, and we found that isolate Lyd4 (*Serratia marcescens*) caused 60% mortality in *L. dispar* larvae. *Erwinia*, a genus containing mostly plant pathogenic species, was also isolated from insect species. İnce et al. (36) reported that an *Erwinia* species isolated from *Thaumetopoea pityocampa* (Lep.: Thaumetopoeidae) was not a pathogenic species for *T. pityocampa*.

Insecticidal activities of the isolates belonging to the genus *Bacillus* (Lyd6, Lyd7, Lyd8, and Lyd9) in *L. dispar* larvae were 90%, 40%, 40%, and 90%, respectively, within 10 days. Sezen et al. (39) reported that *B. thuringiensis* isolated from *Melolontha melolontha* L. (Col.: Scarabaeidae) caused 80% mortality in the larvae of *M. melolontha* within 10 days. In another study, Sezen et al. (44) determined that some *B. thuringiensis* isolates from insects had a 75% insecticidal effect on the larvae of *Amphimallon solstitiale* L. (Col.: Scarabaeidae), another scarab larvae. Our results correspond with these studies. Furthermore, *Bacillus* isolates (Lyd6, Lyd7, Lyd8, and Lyd9) were also used as a crystal-spore mixture in the bioassays. The insecticidal effects of the crystal-spore mixtures of Lyd6 and Lyd9 on *L. dispar* larvae were 100% within 10 days. The activities of the other isolates (Lyd7 and Lyd8) were found to be 40% within 10 days. Lopez-Meza and Ibarra (45) recorded that parasporal crystals of *B. thuringiensis* isolated from living larvae of *Anopheles pseudopunctipennis* in Mexico have no infectivity in 4 species of caterpillar, 3 species of mosquito, and 2 species of beetle. In a previous study, Sezen et al. (44) indicated that the insecticidal effect of *B. thuringiensis* crystals from *Amphimallon solstitiale* was 65% on the same pest. A similar study by Sezen et al. (39) also showed that the insecticidal effect of *B. thuringiensis* crystals from *Melolontha melolontha* was 70% in *M. melolontha*. *B. thuringiensis*, which is a naturally occurring bacterial disease of insects, forms a crystalline, parasporal body composed of protein as an insecticidal crystal protein or δ endotoxins (46). These crystalline inclusions show a wide range of specificity for different insect orders, and so it has a high insecticidal activity on the species of Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Homoptera (47).

B. thuringiensis is now the most widely used biologically produced pest control agent. Worldwide sales of *B. thuringiensis* in 1995 were US$90 million, representing almost 2% of the total global insecticide market. Rowe et al. (48) reported annual worldwide distribution of *B. thuringiensis* as 2.3×10^6 kg. In early 1998, there were nearly 200 registered *B. thuringiensis* products in the United States. Although the use of biological pesticides in agriculture remains significantly behind that of synthetic chemical pesticides, several environmental and safety considerations favor the future development of *B. thuringiensis*. Cry proteins that have been studied thus far are not pathogenic to mammals, birds, amphibians, or reptiles; however, they are very active against specific insects and invertebrate pests. Cry-based pesticides are also generally inexpensive to develop and register (46).

In conclusion, this is the first study on culturable bacteria of *Lymantria dispar* and the pathogenicity of isolated species on the larvae of *L. dispar*. These results will offer useful information for future studies

on the development of bacterial biocontrol agents. Our results indicate that isolates Lyd6 and Lyd9, and the crystal-spore mixture of these isolates, are highly promising as biocontrol agents against *L. dispar*. However, it is important to keep in mind that virulence may differ from species to species or from strain to strain. In addition, the diet of insects and the experimental conditions may affect insecticidal activity.

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