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Isolation, characterization and virulence of bacteria from *Ostrinia nubilalis* (Lepidoptera: Pyralidae)

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Abstract: Isolation, characterization and virulence of the culturable bacteria from entire tissues of larval *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) were studied to obtain new microbes for biological control. A total of 16 bacteria were isolated from living and dead larvae collected from different maize fields in the Eastern Black Sea Region of Turkey. The bacterial microbiota of *O. nubilalis* were identified as *Pseudomonas aeruginosa* (On1), *Brevundimonas aurantiaca* (On2), *Chryseobacterium formosense* (On3), *Acinetobacter* sp. (On4), *Microbacterium thalassium* (On5), *Bacillus megaterium* (On6), *Serratia* sp. (On7), *Ochrobactrum* sp. (On8), *Variovorax paradoxus* (On9), *Corynebacterium glutamicum* (On10), *Paenibacillus* sp. (On11), *Alcaligenes faecalis* (On12), *Microbacterium testaceum* (On13), *Leucobacter* sp. (On14), *Leucobacter* sp. (On15) and *Serratia marcescens* (On16) based on their morphological and biochemical characteristics. A partial sequence of the 16S rRNA gene was also determined to confirm strain identification. The highest insecticidal activities were obtained from *P. aeruginosa* On1 (80%), *Serratia* sp. On7 (60%), *V. paradoxus* On9 (50%) and *S. marcescens* On16 (50%) against larvae 14 days after treatment ($p < 0.05$). Also, the highest activity from previously isolated *Bacillus* species was observed from *Bacillus thuringiensis* subsp. *tenebrionis* Xd3 with 80% mortality within the same period ($p < 0.05$). Our results indicate that *P. aeruginosa* On1, *Serratia* sp. On7, *V. paradoxus* On9, *S. marcescens* On16 and *B. thuringiensis* subsp. *tenebrionis* Xd3 show potential for biocontrol of *O. nubilalis*.

Key words: European corn borer; bacterial microbiota; biocontrol.

Abbreviations: LB, Luria Bertani; OD, optical density.

Introduction

The family Pyralidae includes some of the most damaging species for agricultural plants almost all over the world. Notably, the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae), is a major pest of maize and other crops, such as potato, green pepper, millet, hemp, soybean, cotton, hop, sorghum, cowpea, beans and winter wheat, in Europe, including Turkey and also the other parts of the world (Riba et al. 2005).

According to the Agricultural Control Technical Recommendations from the Ministry of Agriculture, Turkey (2008, Vol. 4, Basak Publisher, Ankara) conventional control of *O. nubilalis* relies to a large extent on the use of foliar chemical insecticides, such as Carbaryl 5%, Profenofos 500 g/L, Thiodicarb 80%, Methomyl 90% and Chlorpyrifos-ethyl 480 g/L. Chemical control is, however, expensive and may have hazardous effects in the environment. In terms of *O. nubilalis*, chemical control is either unsatisfactory owing to their being protected as stem borers or undesirable from an integrated pest management and environmental perspective (Riba et al. 2005). Biological control of this pest is, therefore, an attractive alternative to control *O. nubilalis* since this pest lives in stems.

A number of different phytophagous insects are associated with various symbiont and endosymbiotic bacteria. Associations between organisms and bacteria depend on the host, the symbiont and environmental condition, and may play significant roles in their development, fitness, resistance against enemies, and biological control. The insect gut may provide a suitable habitat for symbiotic bacteria, and these symbioses range from pathogenic to mutualistic, and from facultative to obligate (Kikuchi 2009; Prado & Almeida 2009; Tada et al. 2011). The symbiotic bacteria might be genetically transformed to express insecticidal proteins in the gut to develop novel methods for biological control of pests (Lacey et al. 2007). This provides proof of principle for the use of symbionts as biological control agents (Li et al. 2005). Therefore, until now, scientists have been determined the bacterial microbiota of many pests species in both agriculture and forestry (Kuzina et al. 2001; Demir et al. 2002; Osborn et al. 2002; Sezen et al.

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2004, 2005; Geiger et al. 2009; Gokce et al. 2010; Sevim et al. 2010, 2012).

It is always desirable to search for more effective, pathogenic and safer biocontrol agents against the target pests. For over 50 years, a great number of bacterial species have been isolated from insects, identified, and demonstrated in the laboratory to be pathogenic for various insects (Mostakim et al. 2011). The group of entomopathogenic bacteria includes species with the ability to infect uncompromised healthy insects. This group also includes a large number of opportunistic pathogens which multiply rapidly if they gain access to the hemocoel of stressed insect hosts through wounds or following infection. *Bacillus thuringiensis* is the best-known bacterium which has been used primarily for the control of pests belonging to Diptera, Coleoptera and Lepidoptera (Raddadi et al. 2007). Until now, it has been mainly focused on *B. thuringiensis* for the controlling of *O. nubilalis* and a number of commercial products based on *B. thuringiensis* are being used against this pest worldwide (Lozzia & Manachini 2003). Other bacterial pathogens of *O. nubilalis* are neglected. In this study, we investigated the culturable bacterial microbiota of *O. nubilalis* to discover new bacterial pathogens that can be used as possible biocontrol agents against this pest.

Material and methods

Origin of larvae

O. nubilalis larvae were collected from infested maize fields in the Eastern Black Sea Region of Turkey. Corn stems containing *O. nubilalis* larvae were picked up from different localities between 2008–2010. Collected stems were carefully cut by a scalpel and larvae were removed and put into sterile plastic boxes (25 mm) with ventilated lids. Small corn stems were provided as food and the boxes were immediately transported to the laboratory. Dead and living larvae were separated and living larvae were held at room temperature under 12:12 photoperiod until processing.

Isolation of the culturable bacteria from larvae

Bacterial culture was carried out under aerobic conditions on solid media. Bacteria were isolated from dead and living larvae separately. All dead larvae and 20 living larvae were separately placed into sterile plastic boxes (15 mm) and were surface sterilized with 70% ethanol. Surface-sterilized larval samples were individually homogenized in nutrient broth (Merck, Germany) using a glass tissue grinder. From both mixtures which filtered through two sterile layers of muslin into a sterile 10 mL tube, 10, 50 and 100 µL were plated on nutrient agar (Merck, Germany) and then plates were incubated at 30 °C for 2–3 days. The remaining mixtures were incubated at 30 °C for 5–6 h to increase the number of bacteria which have low concentration. From these mixtures, 10 and 50 µL were plated on nutrient agar and plates were incubated at 30 °C for 2–3 days. Finally, the remaining larval suspensions were heated at 80 °C for 10 min in a water bath to eliminate non-spore-forming organisms, and 100 µL of it were plated on nutrient agar and incubated at 30 °C.

At the end of the incubation period, individual colonies were isolated and distinguished from each other according to their colony characters and colour on the agar medium. The pure bacterial isolates obtained via sub-culturing were identified based on morphological, biochemical and molecular tests.

Identification of the bacterial isolates

Morphological and biochemical properties of the bacterial isolates were determined by three conventional approaches according to *Bergey's Manual of Systematic Bacteriology*, Volumes 1 and 2 (Palleroni 1984; Kandler & Weiss 1986). Gram staining, string test, growth on MacConkey agar and mannitol salt agar, and endospore staining were initially performed for all bacterial isolates. In addition to conventional tests, biochemical characteristics of bacterial isolates were also determined using Analytical Profile Indexes API20E and API50CH panel systems (bioMerieux, France).

16S rRNA gene sequencing

Total genomic DNA was isolated using standard phenol/chloroform procedures for all bacterial isolates (Sambrook et al. 1989). DNA pellets were dissolved in 50 µL of Tris-ethylenediamine tetraacetic acid buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4 °C until use. PCR amplification of the 16S rRNA gene of isolates was selectively amplified from genomic DNA using the following universal primers. UNI16S-L: 5'-ATTCTAGAGTTTGATCAT GGCTCA-3' as forward and UNI16S-R: 5'-ATGGTACCGT GTGACGGGCGGTGTGTA-3' as reverse (William et al. 1991).

16S rRNA gene sequences of the bacterial isolates were amplified in a 50 µL reaction volume containing the following ingredients: 5 µL 10X *Taq* DNA polymerase reaction buffer, 1.5 µL 10 mM dNTP mix, 1.5 µL 10 pmol each of the opposing primers, 1 µL 5 U/µL of *Taq* DNA polymerase (Fermentase), 3 µL 25 mM $MgCl_2$, 2 µL genomic DNA and 34.5 µL dH_2O. PCR conditions were as follows: initial denaturation at 95 °C for 2 min; 30 cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min; and final extension at 72 °C for 5 min. PCR products were separated on 1.0% agarose gel, stained with ethidium bromide and viewed under UV light. After checking PCR products, they were purified using QIAquick PCR purification kit (Qiagen) and sent for sequencing (Macrogen, The Netherlands). The obtained sequences were subjected to BLAST searches (Altschul et al. 1990) using the NCBI GenBank database to determine the similarity among bacterial isolates. Finally, the sequences were used for phylogenetic analysis.

Phylogenetic relationship of the bacterial isolates

The evolutionary relationships of the bacterial isolates and their 40 closely related species were evaluated. Sequences were assembled, edited and aligned with BioEdit (Hall 1999). Sequences obtained from the 16S rRNA gene region were compared with NCBI GenBank accessions using BLAST to confirm isolate identification (Altschul et al. 1990). Cluster analyses of the sequences were performed using BioEdit (version 7.09) with ClustalW followed by neighbour-joining analysis with p-distance method (Saiou & Nei 1987). Analyses were performed with MEGA 5.0 software (Tamura et al. 2011) and the reliability of the dendrogram was tested by bootstrap analysis with 1,000 replicates.

Bioassay

O. nubilalis larvae were collected from different regions of the Eastern Black Sea Region of Turkey. Larvae were removed from corn stems by cutting with a scalpel, and then larvae were carefully placed into sterile plastic boxes (15 mm). Third instars from different areas, which were

Table 1. *Bacillus* species used in this study and their host species.

Isolates	Species	Host	Reference
Ar1	*Bacillus circulans*	*Anoplus roboris* (Suffr.) (Coleoptera: Curculionidae)	Demir et al. (2002)
Ar4	*Bacillus sphaericus*	*Anoplus roboris* (Suffr.) (Coleoptera: Curculionidae)	Demir et al. (2002)
As3	*Bacillus cereus*	*Amphimallon solstitiale* (L.) (Coleoptera: Scarabaeidae)	Sezen et al. (2005)
BnBt	*Bacillus thuringiensis* subsp. *kurstaki*	*Balanius nucum* (L.) (Coleoptera: Curculionidae)	Sezen & Demirbag (1999)
Mm2	*Bacillus thuringiensis*	*Melolontha melolontha* (L.) (Coleoptera: Scarabaeidae)	Sezen et al. (2007)
Mm5	*Bacillus sphaericus*	*Melolontha melolontha* (L.) (Coleoptera: Scarabaeidae)	Sezen et al. (2007)
Mm7	*Bacillus weihenstephanensis*	*Melolontha melolontha* (L.) (Coleoptera: Scarabaeidae)	Sezen et al. (2007)
Xd3	*Bacillus thuringiensis* subsp. *tenebrionis*	*Xyleborus dispar* (L.) (Coleoptera: Scolytidae)	Sezen et al. (2008)
Mnd	*Bacillus thuringiensis* subsp. *kurstaki*	*Malacosoma neustria* (L.) (Lepidoptera: Lasiocampidae)	Kati et al. (2005)
Lyd6	*Bacillus thuringiensis*	*Lymantria dispar* (L.) (Lepidoptera: Lymantriidae)	Unpublished
Lyd7	*Bacillus thuringiensis*	*Lymantria dispar* (L.) (Lepidoptera: Lymantriidae)	Unpublished
Lyd8	*Bacillus thuringiensis*	*Lymantria dispar* (L.) (Lepidoptera: Lymantriidae)	Unpublished
Lyd9	*Bacillus thuringiensis*	*Lymantria dispar* (L.) (Lepidoptera: Lymantriidae)	Unpublished

Table 2. The morphological characteristics of the bacterial isolates.[a]

Isolates	Colony colour	Shape of colonies	Shape of bacteria	Gram stain	Spore stain	Source	Growth in NB
On1	Green	Smooth	Bacillus	–	ND	DL	Turbid
On2	Orange	Smooth	Bacillus	–	ND	AL-80 ℃	Turbid
On3	Light orange	Smooth	Coccobacillus	–	ND	AL-30 ℃	Turbid
On4	Cream	Smooth	Coccobacillus	–	ND	DL	Turbid
On5	Yellow	Smooth	Bacillus	+	–	AL-30 ℃	Turbid
On6	Cream	Rought	Bacillus	+	+	AL-30 ℃	Turbid
On7	Cream	Smooth	Coccobacillus	–	ND	AL-30 ℃	Turbid
On8	Cream	Smooth	Coccobacillus	–	ND	AL-30 ℃	Turbid
On9	Yellow	Smooth	Coccobacillus	–	ND	AL-30 ℃	Turbid
On10	Yellow	Smooth	Bacillus	+	–	AL-30 ℃	Turbid
On11	Cream	Rough	Bacillus	–	+	AL-80 ℃	Turbid
On12	Cream	Smooth	Coccobacillus	–	ND	AL-30 ℃	Turbid
On13	Orange	Smooth	Bacillus	+	–	AL-30 ℃	Turbid
On14	Cream	Filamentous	Bacillus	+	–	AL-30 ℃	Precipitated
On15	Cream	Smooth	Bacillus	+	–	AL-30 ℃	Turbid
On16	Red	Smooth	Coccobacillus	–	ND	DL	Turbid

[a] ND: no data; DL: dead larvae; AL: alive larvae; NB: nutrient broth.

initially segregated, were mixed and individuals then selected at random for use in the bioassays. A total of 16 isolates which were directly isolated from *O. nubilalis* and 13 *Bacillus* isolates which were isolated from different hosts were tested in these experiments (Table 1). Bacterial isolates from stock cultures were transferred to fresh nutrient agar (Merck, Germany) to select single colonies for each isolate. Bacteria from single colonies were inoculated into 5 mL nutrient broth (Merck, Germany) and incubated at 30 ℃ for 25 h. Slow-growing isolates were incubated for 96 h. After the incubation period, cultures were centrifuged at 3,000 rpm for 10 min and pellets were re-suspended in sterile phosphate buffered saline. The optical density of the cell suspension was adjusted to 1.89 at 600 nm (approximately 1.8×10^9 CFU/mL) (Ben-Dov et al. 1995).

The new bacterial isolates of *O. nubilalis* and *Bacillus* isolates were evaluated separately. Corn stems were collected from fields and cut into 10 cm lengths. The pieces were longitudinally divided into two equal parts and a rectangle-shaped hole was made in the middle of both pieces of the divided stems. One mL bacterial suspension for each isolate was put into these holes. After that, larvae were separately put into the contaminated holes. A single European corn borer larva was put into each treated stem and 5 replicate stems were set up for each bacterial treatment. Finally, the second piece of stem was placed over the first and the two halves secured together with a rubber band. All stems were put into plastic boxes (40 mm), and one box was used per isolate. The control group was treated with sterile phosphate buffered saline only. The boxes were incubated at 25 ℃ under 12:12 photoperiod. Insect mortality was recorded 14 days later. All experiments were repeated three times on different days.

Mortalities were corrected according to Abbott's formula (Abbott 1925). The data were subjected to ANOVA and subsequently to LSD multiple comparison test to compare test isolates against the control group and to determine differences among isolates. All analyses were performed using SPSS 16.0 statistical software.

Results

A total of 16 bacterial strains belonging to 13 genera and 14 species were isolated and characterized. Except for *Bacillus megaterium* On6, *Paenibacillus* sp. On11 and *Leucobacter* sp. On14, colony shapes of isolates were smooth. All isolates were bacilli. Six isolates (*Microbacterium thalassium* On5, *B. megaterium* On6, *Corynebacterium glutamicum* On10, *Microbacterium testaceum* On13, *Leucobacter* sp. On14 and *Leucobacter* sp. On15) were Gram positive and the remaining were Gram negative. While *Pseudomonas aeruginosa* On1, *Acinetobacter* sp. On4 and *Serratia marcescens*

Table 3. Biochemical characteristics of the bacterial isolates by conventional methods.[a]

Isolates	Catalase	Oxidase	Starch hydrolysis	String test	Coagulase	MSA	MCA
On1	+	+	–	+	WP	–	+
On2	+	+	WP	+	–	–	+
On3	+	+	WP	+	WP	–	+
On4	+	–	–	+	–	–	+
On5	+	WP	–	–	WP	+	–
On6	+	–	+	–	WP	+	–
On7	+	WP	–	+	–	–	+
On8	+	+	–	+	–	–	+
On9	+	+	–	+	WP	–	+
On10	+	–	–	–	WP	+	–
On11	WP	WP	+	+	–	–	+
On12	+	+	–	+	–	–	+
On13	WP	–	–	–	–	+	–
On14	+	WP	–	–	–	+	–
On15	+	WP	–	–	–	+	–
On16	+	–	+	+	–	–	+

[a] WP: weakly positive; MCA: MacConkey agar; MSA: mannitol salt agar.

Table 4. Biochemical characteristics of the G^- bacterial isolates based on API20E.[a]

	Isolates										
Tests	1	2	3	4	7	8	9	11	12	15	16
β-Galactosidase	–	–	+	–	+	–	–	+	–	WP	+
Arginine dihydrolase	+	–	–	–	+	–	–	–	–	–	–
Lysine decarboxylase	+	–	–	–	+	–	–	–	–	–	–
Ornithine decarboxylase	–	–	–	–	+	–	–	–	–	–	+
Citrate utilization	+	–	–	–	+	–	+	–	+	–	+
H_2S	–	–	–	–	–	–	–	–	–	–	–
Urease	–	–	–	–	+	+	–	–	–	–	–
Tryptophane deaminase	–	–	–	–	–	–	–	–	–	–	–
Indole	–	–	+	–	–	–	–	–	–	–	–
Acetoin	–	WP	WP	WP	+	WP	–	–		–	+
Gelatinase	+	–	+	–	+	–	+	–	–	+	+
Glucose	+	–	WP	–	+	–	–	+	–	WP	+
Mannitol	–	–	–	–	+	–	–	+	–	–	+
Inositol	–	–	–	–	WP	–	–	–	–	–	–
Sorbitol	–	–	–	–	+	–	–	–	–	–	+
Rhamnose	–	WP	–	–	WP	–	–	WP	–	–	–
Saccharose	–	–	WP	–	+	–	–	+	–	WP	+
Melibiose	+	–	–	–	+	–	–	+	–	WP	+
Amygdalin	–	–	WP	–	+	–	–	+	–	–	+
Arabinose	+	–	–	–	+	WP	–	+	–	WP	–
NO_2	+	–	–	–	WP	–	+	–	–	+	–
Reduction to N_2 gas	–	+	+	+	+	WP	–	WP	WP	–	+

[a] WP: weakly positive.

On16 were obtained from dead larvae, other isolates were isolated from living larvae (Table 2).

All isolates were catalase positive. Oxidase and starch hydrolysis tests varied depending on the isolate. String test results were almost compatible with Gram staining. All isolates were negative in terms of coagulase although some of these were weakly positive (Table 3). API test results are listed in Tables 4 and 5.

Table 6 summarizes the results of the 16S rRNA gene sequence analysis for the bacterial isolates. Approximately 1,300 bp fragment of the 16S rRNA gene was sequenced and used for BLAST search. BLAST results and the most closely related species are presented in Table 6. Based on the conventional tests, API20E, API50CH and the 16S rRNA sequencing, isolates were identified as *Pseudomonas aeruginosa* (On1), *Brevundimonas aurantiaca* (On2), *Chryseobacterium formosense* (On3), *Acinetobacter* sp. (On4), *Microbacterium thalassium* (On5), *Bacillus megaterium* (On6), *Serratia* sp. (On7), *Ochrobactrum* sp. (On8), *Variovorax paradoxus* (On9), *Corynebacterium glutamicum* (On10), *Paenibacillus* sp. (On11), *Alcaligenes faecalis* (On12), *Microbacterium testaceum* (On13), *Leucobacter* sp. (On14), *Leucobacter* sp. (On15) and *Serratia marcescens* (On19). These identifications were also confirmed by phylogenetic analysis of the bacterial isolates and their closely related species based on the 16S rRNA sequence (Fig. 1).

Mortality differed for *O. nubilalis* larvae exposed to bacterial isolates of *O. nubilalis* origin ($F = 3.31$,

Table 5. Biochemical characteristics of the G^+ bacterial isolates based on API50CH.[a]

Tests	Isolates											
	5		6		10		13		14		15	
	Test time											
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Glycerol	WP	WP	WP	+	–	–	WP	+	–	–	–	–
Erythritol	–	WP	–	–	–	–	–	–	–	–	–	–
D-Arabinose	–	WP	–	–	–	–	–	–	–	–	–	–
L-Arabinose	WP	+	WP	+	–	–	+	+	–	–	–	–
D-Ribose	WP	+	WP	+	+	+	–	–	–	+	–	–
D-Xylose	–	WP	WP	+	–	–	+	+	–	–	–	–
L-Xylose	–	–	–	–	–	–	–	–	–	–	–	–
D-Adonitol	–	–	–	–	–	–	–	–	–	–	–	–
Methyl-β-D-xylopyranoside	WP	WP	–	–	–	–	–	–	–	–	–	–
D-Galactose	+	+	WP	WP	–	–	+	+	–	–	–	–
D-Glucose	+	+	+	+	WP	+	+	+	–	–	–	WP
D-Fructose	+	+	+	+	WP	+	+	+	–	+	–	WP
D-Mannose	–	–	–	–	WP	+	+	+	–	–	–	–
L-Sorbose	–	–	–	–	–	–	–	–	–	–	–	–
L-Rhamnose	–	–	–	–	–	–	–	–	–	–	–	–
Dulcitol	–	–	–	–	+	+	–	–	–	–	–	–
Inositol	–	–	–	WP	+	+	–	–	–	–	–	–
D-Mannitol	+	+	+	+	+	+	+	+	–	–	–	WP
D-Sorbitol	–	–	–	–	–	–	–	–	–	–	–	–
Methyl-α-D-mannopyranoside	–	–	–	–	–	–	–	–	–	–	–	–
Methyl-α-D-glucopyranoside	–	WP	–	–	–	–	–	–	–	–	–	–
N-Acetylglucosamine	–	–	WP	+	–	–	–	–	–	–	–	–
Amygdalin	WP	+	WP	+	–	–	–	–	–	–	–	–
Arbutin	WP	+	WP	+	+	+	–	–	–	WP	–	–
Esculin-ferric citrate	+	+	+	+	+	+	+	+	–	+	–	–
Salisin	+	+	WP	+	+	+	–	–	+	+	–	–
D-Celiobiose	+	+	WP	+	–	–	+	+	–	–	–	–
D-Maltose	+	+	+	+	–	–	+	+	–	–	–	–
D-Lactose (bovine origin)	WP	+	–	–	–	–	–	–	–	–	–	–
D-Melibiose	WP	+	WP	+	–	–	–	–	–	–	–	–
D-Saccharose	WP	+	+	+	+	+	+	+	–	–	–	WP
D-Trehalose	+	+	+	+	–	–	–	WP	–	–	–	–
Inulin	–	–	WP	+	–	–	–	–	–	–	–	–
D-Melezitose	+	+	–	–	–	–	WP	+	–	–	–	–
D-Raffinose	WP	+	+	+	–	–	–	–	–	–	–	–
Starch	–	–	+	+	–	–	–	–	–	–	–	–
Glycogen	–	–	+	+	–	–	–	–	–	–	–	–
Xylitol	–	–	–	–	–	–	–	–	–	–	–	–
Gentiobiose	–	WP	+	+	–	–	–	–	–	–	–	–
D-Turanose	WP	+	WP	–	–	–	WP	+	–	–	–	–
D-Lyxose	–	–	–	–	–	–	–	WP	–	–	–	–
D-Tagatose	–	–	–	–	–	–	–	–	–	–	–	–
D-Fucose	–	–	–	–	–	–	–	–	–	–	–	–
L-Fucose	–	–	–	–	–	–	–	–	–	–	–	–
D-Arabitol	–	–	–	–	+	+	–	–	–	–	–	–
L-Arabitol	–	–	–	–	–	–	–	–	–	–	–	–
Potassium gluconate	–	WP	–	WP	–	–	WP	+	–	+	–	–
Potassium 2–ketogluconate	WP	+	–	WP	–	+	–	–	–	–	–	–
Potassium 5–ketogluconate	–	–	–	–	–	–	–	–	–	–	–	–

[a] WP: weakly positive.

$df = 16$, $p < 0.05$) and all isolates were significantly different from the control group, except for *Acinetobacter* sp. (On4), *B. megaterium* (On6), *Ochrobactrum* sp. (On8), *C. glutamicum* (On10), *Paenibacillus* sp. (On11), *A. faecalis* (On12), *M. testaceum* (On13), *Leucobacter* sp. (On14) and *Leucobacter* sp. (On15) ($F = 3.31$, $df = 16$, $p < 0.05$). The highest insecticidal effects were obtained from *P. aeruginosa* On1 (80%), *Serratia* sp. On7 (60%), *V. paradoxus* On9 (50%) and *S. marcescens* On16 (50%) 14 days after inoculation ($F = 3.31$, $df = 16$, $p < 0.05$). Pathogenicity of the remaining isolates ranged from 40% to 10% (Fig. 2).

For the *Bacillus* species, all isolates produced different mortality values in comparison to each other ($F = 9.13$, $df = 13$, $p < 0.05$) and were different from the control group ($F = 9.13$, $df = 13$, $p < 0.05$). The highest mortality was obtained from *B. thuringiensis* subsp. *tenebrionis* Xd3 with 80% mortality value against larvae 14 days after inoculation ($F = 9.13$, $df = 13$, $p < 0.05$). Pathogenicity of the remaining *Bacillus* species ranged from 60% to 20% (Fig. 3).

Table 6. Suggested identification of bacterial isolates according to the partial 16S rRNA gene sequence.

Isolates / GenBank	Suggested identification from GenBank	Query coverage (%)	16S rRNA similarity (%)	Accession numbers
On1 / HQ377320	*Pseudomonas aeruginosa* AU2039B	100	99	AY486356
	Pseudomonas aeruginosa MZA-85	99	99	HQ023428
On2 / HQ377321	*Brevundimonas* sp. DNA	100	99	AJ227796
	Brevundimonas aurantiaca CB-R	100	99	NR028889
On3 / HQ377322	*Chryseobacterium formosense* CC-H3–2	97	98	AY315443
	Chryseobacterium formosense	97	98	AJ715377
On4 / HQ377323	*Acinetobacter* sp. A1PC16	100	99	FN298236
	Acinetobacter sp. G30	100	99	EF204258
On5 / HQ377324	*Microbacterium thalassium*	100	98	AM181507
	Microbacterium flavescens 3373	100	97	EU714363
	Microbacterium paraoxydans 76	100	97	EU714377
On6 / HQ377325	*Bacillus megaterium* QM B1551	100	100	CP001983
	Bacillus megaterium IMAUB1027	100	100	FJ641033
On7 / HQ377326	*Pseudomonas fluorescens* YR20	99	99	HM224401
	Serratia marcescens HO2–A	99	99	AJ297950
	Serratia nematodiphila DZ0503SBSH1–2	99	99	EU914257
On8 / HQ377327	*Ochrobactrum pseudogrignonense* 5JN2	100	98	GU991856
	Ochrobactrum pseudogrignonense BIHB	100	97	FJ859687
	Ochrobactrum sp. 15	100	97	EF537010
On9 / HQ377328	*Variovorax paradoxus* rif200835	99	98	FJ527675
	Variovorax sp. CN3b	99	98	GQ332345
	Variovorax sp. CYEB-15	99	98	FJ422402
On10 / HQ377329	*Corynebacterium glutamicum* ATCC 13032	100	98	BA000036
	Corynebacterium glutamicum TCCC27026	100	98	EU231610
On11 / HQ377330	*Paenibacillus amylolyticus*	100	99	AB115960
	Paenibacillus sp. IDA5358	100	99	AY289507
On12 / HQ377331	*Alcaligenes faecalis* SP03	100	99	EF427887
	Alcaligenes faecalis AU02	100	99	HM145896
On13 / HQ377332	*Microbacterium* sp. Acj 118	100	98	AB480762
	Microbacterium testaceum 343	100	99	EU714365
	Microbacterium testaceum CE648	100	98	AF474330
On14 / HQ377333	*Leucobacter* sp. clone A4H6M8	99	98	GQ206322
	Leucobacter iarius 40	100	98	AM040493
	Leucobacter komagatae	100	98	AJ746337
	Leucobacter luti	100	98	AM072819
On15 / HQ377334	*Leucobacter tardus*	100	99	AM940158
	Leucobacter sp. BK-26	98	98	GU564354
	Leucobacter sp. M1–8	100	96	GQ352403
On16 / HQ377335	*Serratia marcescens* XJ-01	100	99	FJ530951
	Serratia marcescens HL1	99	99	EU371058

Discussion

There is an increasing interest in finding more pathogenic and effective bacterial control agents against various insect pests to reduce the damaging effects of chemical insecticides on the environment, and to provide sustainable and ecologically acceptable methods for controlling insect pests in both agriculture and forest ecosystems. In this study, we investigated the culturable bacterial microbiota of *O. nubilalis* to identify new candidate organisms as a possible biocontrol agent against this pest. Greater bacterial diversity was detected in the culturable microbiota from living rather than from dead larvae.

The association of *Pseudomonas aeroginosa* (Li et al. 2005), *Acinetobacter* sp. (Sevim et al. 2010), *Bacillus megaterium* (Sevim et al. 2010), *Paenibacillus* sp. (Gokce et al. 2010), *Alcaligenes faecalis* (Osborn et al. 2002), *Serratia marcescens* (Rani et al. 2009), *Leucobacter* sp. (Murrell et al. 2003), *Variovorax paradoxus* (Dillon et al. 2008), *Corynebacterium glutamicum* (Mrazek et al. 2008) and *Ochrobactrum* sp. (Volf et al. 2002) with insects has previously been demonstrated. However, to our knowledge, this is the first documentation of *Brevundimonas aurantiaca*, *Chryseobacterium formosense*, *Microbacterium thalassium* and *Microbacterium testaceum* species from any insect. Identification of bacterial isolates at the species level using classical and molecular techniques is sometimes difficult. Further studies would be required to identify these isolates beyond the genus level. However, we did not conduct further identification studies for the isolates, which were identified at the genus level and were not insecticidal against *O. nubilalis*.

Lynch et al. (1976) isolated and identified bacterial isolates from eggs and the first instar *O. nubilalis*. They also performed pathogenicity assay against *O. nubilalis*. In total, they isolated 14 bacteria including *Bacillus thuringiensis* var. *kurstaki*, *B. cereus*, *B. megaterium*, *Acinetobacter* sp., *Erwinia herbicola*, *Enterobacter cloacae*, *Serratia marcescens*, *Pseudomonas* sp., *Xanthomonas* sp., *Enterobacter* sp., *Klebsiella* sp., *Micrococcus luteus*, *Streptococcus* sp. and *S. faecalis*. In addition, Belda at al. (2011) studied the microbial diversity in the midguts of field and lab-reared populations of *O. nubilalis*. In their study, a great amount

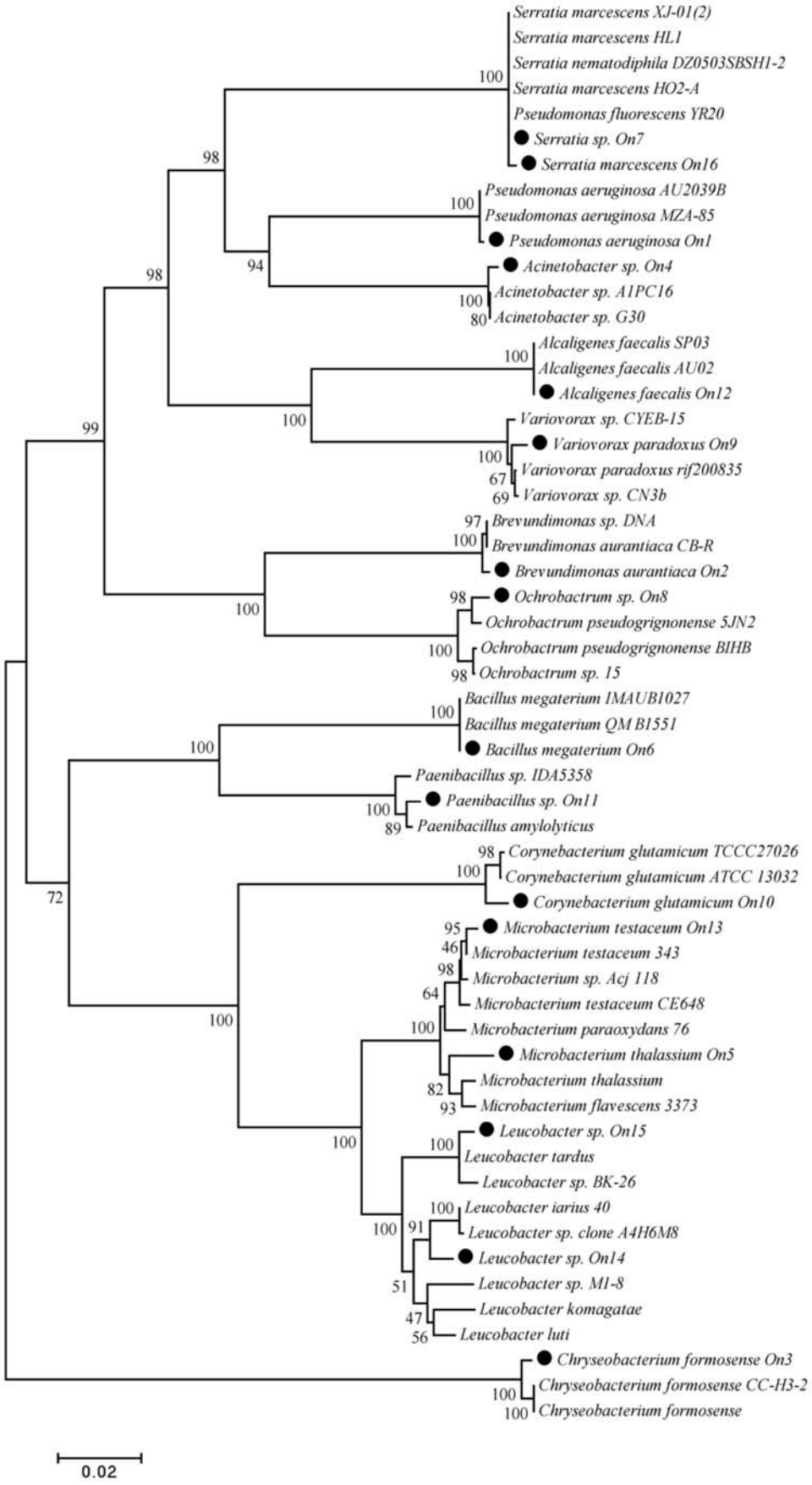

Fig. 1. Phylogenetic analysis of bacterial isolates and their 40 closely related species based on the 16S rRNA sequence. Approximately 1,300 bp fragment of the 16S gene was used. Neighbour-joining analysis with p-distance method was used to construct the dendrogram. Bootstrap values shown next to nodes are based on 1,000 replicates. Bootstrap values C $\geq$ 70% are labelled. *O. nubilalis* isolates were indicated with black dots. The scale on the bottom of the dendrogram shows the degree of dissimilarity.

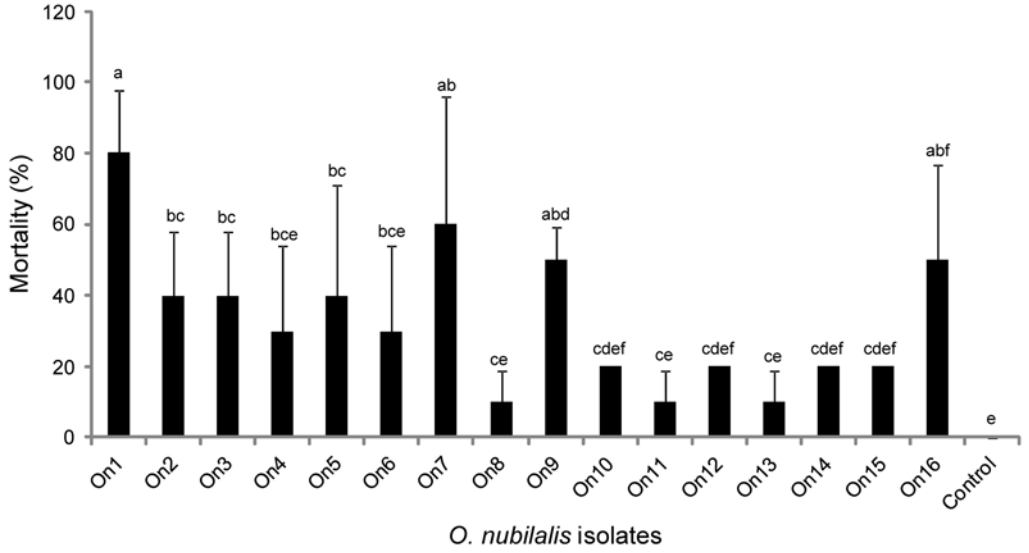

Fig. 2. Mortality of the third instar *O. nubilalis* larvae 14 days after treatment with *O. nubilalis* isolates. Mortalities were corrected using Abbott's formula. Different letters indicate significant differences between isolates according to the LSD multiple comparison test ($p < 0.05$). Bars show standard deviation. On1: *Pseudomonas aeroginosa*, On2: *Brevundimonas aurantiaca*, On3: *Chryseobacterium formosense*, On4: *Acinetobacter* sp., On5: *Microbacterium thalassium*, On6: *Bacillus megaterium*, On7: *Serratia* sp., On8: *Ochrobactrum* sp., On9: *Variovorax paradoxus*, On10: *Corynebacterium glutamicum*, On11: *Paenibacillus* sp., On12: *Alcaligenes faecalis*, On13: *Microbacterium testaceum*, On14: *Leucobacter* sp., On15: *Leucobacter* sp., On16: *Serratia marcescens*.

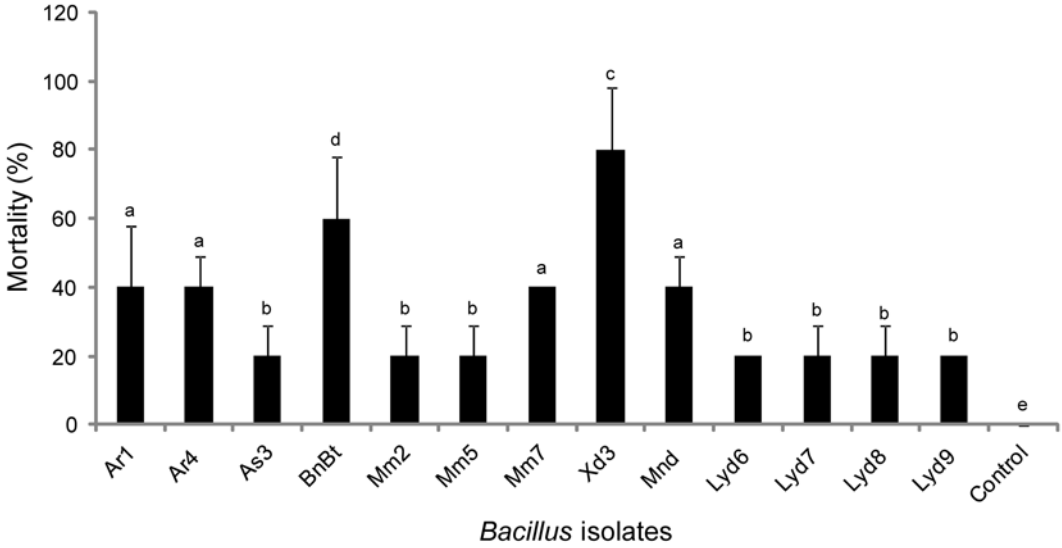

Fig. 3. Mortality of the third instar *O. nubilalis* larvae 14 days after treatment with *Bacillus* isolates. Mortalities were corrected using Abbott's formula. Different letters indicate significant differences between isolates according to the LSD multiple comparison test ($p<0.05$). Bars show standard deviation. Ar1: *Bacillus circulans*, Ar4: *Bacillus sphaericus*, As3: *Bacillus cereus*, BnBt: *Bacillus thuringiensis*, Mm2: *Bacillus thuringiensis*, Mm5: *Bacillus sphaericus*, Mm7: *Bacillus weihenstephanensis*, Xd3: *Bacillus thuringiensis* subsp. *tenebrionis*, Mnd: *Bacillus thuringiensis*, Lyd6: *Bacillus thuringiensis*, Lyd7: *Bacillus thuringiensis*, Lyd8: *Bacillus thuringiensis*, Lyd9: *Bacillus thuringiensis*.

of microbial diversity was detected and *Staphylococcus warneri* and *Weissella paramesenteroides* were dominant species. In our study, however, *B. aurantiaca* (On2), *C. formosense* (On3), *M. thalassium* (On5), *V. paradoxus* (On9), *C. glutamicum* (On10), *Paenibacillus* sp. (On11), *A. faecalis* (On12), *M. testaceum* (On13), *Leucobacter* sp. (On14) and *Leucobacter* sp. (On15) were reported for the first time as bacterial microbiota of *O. nubilalis*. In the studies of Lynch et al. (1976), *B. thuringiensis* var. *kurstaki* was pathogenic to *O. nubilalis* larvae and *B. megaterium* to eggs. However, in our study, we were able to show that many other bacterial species are also pathogenic to *O. nubilalis* and may be useful biocontrol agents (Figs 2 and 3).

The highest (80%) insecticidal effect was obtained from *P. aeruginosa* (On1). To date, *P. aeroginosa* has been isolated from, and been shown to be pathogenic to, several insect species (Banerjee & Dangar 1995; Inglis et al. 2000; Osborn et al. 2002). Banerjee & Dangar (1995) showed that *P. aeroginosa* is pathogenic to red palm weevil (*Rhynchophorus ferrugineus* (Olivier)) and

caused 69% larval mortality. Inglis et al. (2000) also observed that *Pseudomonas* spp. isolates were pathogenic to south-western corn borer (*Diatraea grandiosella* (Dyar)) larvae under controlled laboratory conditions. In addition, Osborn et al. (2002) reported 60–93.3% mortality from *P. aeroginosa* against *Hylesia metabus* (Cramer) larvae.

S. marcescens causes disease both in plants and in a wide range of invertebrate and vertebrate hosts. This species is one of the most well-known bacterial pathogen of insects. Using *S. marcescens*, Lauzon et al. (2003) obtained 50% mortality against *Rhagoletis pomonella* (Walsh) flies. Bahar & Demirbag (2007) found that *S. marcescens* Ol13 caused 65% mortality in *Oberia linearis* (L.) larvae. In addition, Sezen & Demirbag (1999) reported that *S. marcescens*, isolated from *Balanninus nucum*, caused 100% mortality in *B. nucum* larvae within 3 days. Gokce et al. (2010) also showed that *S. marcescens* Rb2 has 75% mortality against larvae of *Rhynchites bacchus* (L.). Our results correspond with these studies and we found that *Serratia* sp. On7 and *S. marcescens* On16 produced 60 and 50% mortality against *O. nubilalis* larvae, respectively.

Several *Paenibacillus* species are associated with insects, with *P. popilliae*, *P. lentimorbus*, *P. larvae*, *P. apiarus* and *P. alvei* being pathogenic to several insect species, whereas others are facultative pathogens (Pettersson et al. 1999). Among these, *P. popilliae* is a highly fastidious species that is the primary etiologic agent of the so-called milky diseases of scarab larvae (Federici 2007). Although certain *Paenibacillus* species are known as definitive insect pathogens, in this study, we just observed 10% mortality value from *Paenibacillus* sp. On11, suggesting this isolate may not be insect pathogen.

Up to now, *V. paradoxus* has only been isolated from grasshopper (Dillon et al. 2008). In addition, insect pathogenic activity has never been demonstrated. We isolated *V. paradoxus* from *O. nubilalis* and obtained 50% mortality in the larval bioassay. However, detailed studies such as dose-mortality response testing need to be done to further quantify the relative virulence of the different bacterial isolates.

Many different *Bacillus* species have been isolated from dead or living insects. The *Bacillus* species commonly recognized as definitive insect pathogens, however, are *B. popilliae* (formerly *B. popillae*), *B. lentimorbus*, *B. larvae*, *B. thuringiensis*, and certain strains of *B. sphaericus* (Stahly et al. 1992). The spore-forming bacilli are viewed as having the highest potential for use in the management of insect pest populations, and these organisms have received the greatest commercial attention. There are currently 410 registered formulations of *B. thuringiensis* and 6 registered formulations of *B. lentimorbus* and *B. popillae* approved for use in the United States against insect pests (Aronson et al. 1986). In this study, we tested 13 *Bacillus* species against *O. nubilalis* and obtained the highest activity from *B. thuringiensis* subsp. *tenebrionis* Xd3. This isolate has been also previously shown that it has a good insecticidal activity (100%) against *Agelastica alni* (L.), *Amphimallon solstitiale* (L.), *Leptinotarsa decemlineata* (Say.) and *Melolontha melolontha* (L.) (Sezen et al. 2008). These results mean that *B. thuringiensis* subsp. *tenebrionis* Xd3 should be a valuable biocontrol agent in Black Sea Region of Turkey against a number of insect pests.

In conclusion, we determined the culturable bacterial microbiota of *Ostrinia nubilalis* collected from the Eastern Black Sea Region of Turkey and tested these isolates and 13 *Bacillus* species against the target pest. Our results indicate that *P. aeruginosa* On1, *Serratia* sp. On7, *V. paradoxus* On9, *S. marcescens* On16 and *B. thuringiensis* subsp. *tenebrionis* Xd3 in particular may be good candidates for further investigation as a possible microbial control agent against *O. nubilalis*. However, further studies are needed to determine the effectiveness of these isolates in the field. Additionally, mass production and formulation studies are also warranted. Moreover, ecological and environmental risk assessments, such as toxicity tests against beneficial insects, humans and other animals are also required.

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