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Determination of the bacterial flora as a microbial control agent of *Toxoptera aurantii* (Homoptera: Aphididae)

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Abstract: *Toxoptera aurantii* (Homoptera: Aphididae) is one of the most important pests of many agricultural plants such as camellia, cocoa and coffee worldwide. The culturable bacterial flora of the pest was determined to find new microbes that can be used as biocontrol agent against *T. aurantii*. A total of six bacteria were isolated and identified by a variety of morphological, physiological, biochemical and molecular tests. In addition, an approximately 1,150 bp fragment of the 16S rRNA gene region was sequenced to verify isolate identification. According to the identification studies, the isolates were identified as *Bacillus tequilensis* Cb1, *Chryseobacterium stagni* Cb2, *Pseudomonas flourescens* Cb3, *Rahnella aquatilis* Cb4, *Staphylococcus* sp. Cb5 and Cb6. Pathogenicity of the bacterial isolates were carried out against the last instar nymphs of *T. aurantii*. The highest activity was obtained from *Pseudomonas flourescens* Cb3 with 50% mortality within 10 days after application ($p<0.05$). Mortalities of other treatments ranged from 6.6 to 20%. The results presented here show that *Pseudomonas flourescens* Cb3 appears to be a significant candidate as a possible biocontrol agent against *T. aurantii* and should be beneficial in the future biocontrol programs of the pest.

Key words: bacterial flora; black citrus aphid; *Pseudomonas flourescens*; pathogenicity.

Introduction

Toxoptera aurantii (Homoptera: Aphididae) is a polyphagous species with a worldwide distribution and it has over 120 hosts including camellia, cocoa and coffee, etc. (Carver 1978). It has been considered to be one of the most destructive pests of tea plants in the Eastern Black Sea Region of Turkey. In addition to its direct damage to host plants, aphids vector many plant diseases, such as citrus tristeza virus and coffee ringspot virus, which cause reasonably greater losses than caused by direct feeding injury. This is often the most damaging feature of an aphid infestation (Rao & Capoor 1976; Blackman & Eastop 2000; Dadmal et al. 2000;). Separately, the excreted honeydew by *T. aurantii* on the leaves may be colonized by mildew, which reduces photosynthesis (Han & Chen 2002).

There are a few methods that are being used for controlling *T. aurantii*. Citrus inter-planted with tea encourages buildup of indigenous aphidophagous parasitoids, which are natural enemies of aphids. Removing of infested shoots is also recommended to control this pest in tea gardens (Hazarika et al. 2001). Although there are many natural enemies of *T. auranti*, such as predators, parasites and fungi (Firempong & Kumar 1975), none of them are currently being used for controlling this pest as agricultural practice. In addition to these control methods, according to the 2010 report of the Ministry of Agriculture of Turkey entitled "Control of citrus diseases and pests" (Ankara, 43 pp; in Turkish), some chemical substances such as Pirimicarb 50%, Pymetrozine 25%, and Spirotetramat 100% have been also used for controlling *T. aurantii*. However, these chemicals have undesirable side effects on the environment, human and plants. There is therefore still a need to find more effective and safe biocontrol agent against *T. aurantii*.

Entomopathogenic bacteria are capable of causing disease in insects; however, they do not harm mammals, other animals and plants. They are generally considered to be less toxic to the environment and can be developed, registered and integrated quickly and cheaply into pest management system with respect to biological control. Hence, environmentally compatible pest-control measures have encouraged the interest in bacterial insect pathogens as biological control agent (Bucher 1981; Thiery & Frachon 1997). In the past 50 years a great number of bacteria have been isolated, classified, and demonstrated in the laboratory to be pathogenic for various insects. Many of the insect-pathogenic bacteria occur in the families Pseudomonadaceae, Enterobacteriaceae, Lactobacillaceae, Micrococcaceae, and Bacillaceae (Bulla et al. 1975). Whereas most of the focus on bacterial insecticides has been on spore-forming bacilli, especially *Bacillus thuringiensis* that has been utilized to control many Dipteran, Coleopteran and Lipedopteran insects, other bacteria continue to show potential to control insect pests (Burges 1982). Up to

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now, many scientists have determined the bacterial flora of many insect species having importance in agriculture and forestry, and tested their insecticidal activities against a number of pest species (Sezen et al. 2004; Sezen & Demirbag 2006; Yilmaz et al. 2006; Bahar & Demirbag 2007; Gokce et al. 2010; Sevim et al. 2010).

Although *T. aurantii* is an important pest species of many agricultural plants worldwide, bacterial pathogens of this pest were neglected. In the present study, we isolated the culturable bacteria from this pest and characterized them in detail by a combination of morphological, physiological, biochemical and molecular techniques. In addition, we determined the insecticidal activity of the bacterial isolates against the last instar nymphs of the pest as a possible biocontrol agent. This is the first report indicating isolation of bacteria from *T. aurantii* and their pathogenicity against to the pest.

Material and methods

Collection of nymph samples

Toxoptera aurantii nymphs were collected from the infested tea gardens from the vicinity of Rize, Turkey, in May of 2010 and 2011. Nymphs were carefully taken by a soft paintbrush into plastic boxes (10 mm) with ventilated lids to allow airflow until they were transported to the laboratory. During this time, they were feed by fresh tea leaves. When necessary, whole infested shoots were taken into plastic boxes (10 mm) and transported to the laboratory. They were fed by fresh tea leaves at room temperature under 12:12 photoperiod until bacterial isolation was done. Tea leaves used as diet were washed with sterile distilled water two or three times before use in feeding.

Isolation of bacteria

Collected nymphs were macroscopically inspected with respect to dead, diseased and healthy nymphs, and healthy and dead nymphs were separated and used for the bacterial isolation. The insects were washed with ethanol (70%) three times to prevent possible contamination. After that, 50 surface-sterilized larvae were homogenized in nutrient broth using a glass tissue grinder. Then, two methods were used for isolation. At the first one, nymph suspensions were diluted from 10^{-1} to 10^{-5} and they were plated on nutrient agar followed by incubation at 30 °C for 2–3 days. At the second one, the diluted nymph suspensions were heated at 80 °C for 10 min to eliminate non-spore forming bacteria (Thiery & Frachon 1997). After that, heated suspensions were plated on nutrient agar and incubated at 30 °C for 2–3 days. At the end of the incubation period, plates were examined and bacterial colonies were selected based on their color and morphology. In addition, bacterial colonies were counted to calculate the number of bacteria per nymph. Finally, selected colonies were purified, and identified by a variety of tests. Purified bacteria were stocked at −80 °C until use.

Morphological characterization of the bacterial isolates

Morphological, biochemical and physiological properties of the bacterial isolates were determined according to Bergey's Manual of Systematic Bacteriology, Volumes 1 and 2 (Palleroni 1984; Kandler & Weiss 1986). Stock cultures were plated out on nutrient agar plates and single colonies were picked and sub-cultured. Colony morphologies of the bacterial isolates were examined under stereomicroscope. Gram staining was performed according to the method of Claus (1992). Endospore staining was performed according to the procedure described by Prescott et al. (1996). Capsule staining was performed by negative staining. The motility of isolates was determined using a semisolid medium (Soutourina et al. 2001). The pH, temperature and NaCl tolerance of the bacterial isolates were determined in Luria-Bertani broth for physiological characterization.

Biochemical characterization of the bacterial isolates

Biochemical characterization of bacterial isolates was determined whether isolates produce various enzymes and products by plating and inoculation them into different media including different special features. Catalase, oxidase, nitrate reduction, starch hydrolysis, methyl red test, voges proskauer, citrate, gelatin hydrolysis, coagulase and Kligler iron agar tests were initially used to identify bacterial isolates according to the Bergey's Manual of Systematic Bacteriology, Volumes 1 and 2 (Palleroni 1984; Kandler & Weiss 1986). In addition to these conventional tests, API 50CH test strips were used for further characterization of the isolates.

API 50CH bacterial identification system

API tests were performed according to the recommendations of the manufacturer (bioMerieux, France) with a few small modifications. Stock cultures were plated out on nutrient agar plates and single colonies were picked and diluted in API 50 CHB/E medium. The amount of bacteria was adjusted to 1 McFarland and 200 µL from these bacterial suspensions were poured into the starting well of the panels. In order to prevent contamination from air, wells were filled with mineral oil and then the panels were incubated at 30 °C for 24–48 h. The results of the tests were evaluated according to the computer-based program 'IdBact v. 1.1, G. Kronvall, with Matrix for API20E from bioMerieux, France'.

16S rRNA gene sequencing

For PCR, genomic DNA was extracted using a standard phenol and chloroform protocol (Sambrook et al. 1989). DNA pellets were dissolved in 50 µL of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and isolated DNAs were stored at −20 °C until use.

Approximately 1,400 bp fragment of the 16S rRNA gene was amplified from all bacterial isolates using primers of UNI16S-L (5'-ATTCTAGAGTTTGATCATGGCTCA-3') and UNI16S-R (5'-ATGGTACCGTGTGACGGGCGG TGTGTA-3') (William et al. 1991). The reaction mixture of 50 µL consisted of 1.5 µL 10 mM dNTP mix, 1.5 µL 10 pmol each of the opposing amplification primers, 1 µL 5 U/µL of *Taq* DNA polymerase (Fermentase), 3 µL $MgCl_2$, 5 µL *Taq* DNA polymerase reaction buffer, 2 µL genomic DNA, and 34.5 µL dH_2O. PCR amplifications were performed by Eppendorf Mastercycler Gradient (Hamburg, Germany). Amplification was done by initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing temperature of primers was 54 °C for 1 min and extension at 72 °C for 1 min. The final extension was conducted at 72 °C for 10 min. PCR products were separated on 1.0% agarose gels, stained with ethidium bromide and viewed under the UV light. After checking PCR products, they were sent to MACROGEN (The Netherlands) for sequencing. The obtained sequences were used to perform BLAST searches (Altschul et al. 1990) using the GenBank database (Benson et al. 2012).

Table 1. The morphological characteristics of the bacterial isolates.

Isolate number	Cb1	Cb2	Cb3	Cb4	Cb5	Cb6
Color of colonies	Cream	Orange	Cream	Cream	Cream	Cream
Shape of colonies	Round-mucoid	Smooth	Smooth	Smooth	Smooth	Smooth
Shape of bacteria	Bacil	Bacil	Bacil	Bacil	Coccus	Coccus
Gram stain	+	–	–	–	+	+
Spore stain	+	–	–	–	–	–
Spore form	Central	ND[a]	ND	ND	ND	ND
Capsule	–	–	–	–	–	–
Motility	+	–	–	–	–	–
Turbidity[b]	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid

[a] ND means no data.
[b] Turbidity when grown in nutrient broth.

Phylogeny

Phylogenetic analysis of the bacterial isolates with their 14 closely related species were evaluated. 16S rRNA sequences were assembled and edited with BioEdit (Hall 1999) and aligned. Cluster analyses of the sequences was performed using BioEdit (version 7.09) with ClustalW followed by p-distance method (Kimura 1980) with neighbor-joining analysis on aligned sequences with MEGA 5.0 software (Tamura et al. 2011). Alignment gaps were treated as missing data. Reliability of dendrogram was tested by bootstrap analysis with 1,000 replicates using MEGA 5.0.

Bioassay

Bacterial isolates were incubated at 30 °C for 18 h (72 h for *Bacillus* for sporulation) in nutrient broth. After incubation, the density of cells was adjusted to 1.89 at optical density OD_{600} by adding sterile phosphate-bufferred saline and 5 mL of the culture was centrifuged at 4,000 rpm for 10 min (Ben-Dov et al. 1995). The pellet was re-suspended in 5 mL of sterilized phosphate-buffered saline and it was used for bioassays (Moar et al. 1995).

T. aurantii nymphs were collected from the infested tea gardens during August of 2011, Rize, Turkey. Collected specimens were brought to the laboratory and waited for 2–3 days so that nymphs were acclimated to the laboratory. Freshly collected tea leaves were used as diet in bioassay. Collected leaves were washed with sterile distilled water 2–3 times before use in bioassay. To prepare experimental setup, 1% agar was prepared, poured into plastic petri dishes (15 mm), and waited for 2 days to check possible contamination. After that, freshly collected tea leaves were contaminated with 1 mL of the bacterial solutions prepared as described above and stalks of the contaminated leaves were stick into agar to prevent dry out of leaves and to provide standing upright of them. The sterile phosphate-buffered saline was used in the control group. Finally, ten last instar nymphs for each replicate were carefully placed on each contaminated leave by a soft paintbrush, and plates were incubated at 25±2 °C under 12:12 photoperiod. All experiments were repeated three times on different occasions. The mortality of insects was recorded at 10^{th} day. Mortalities were corrected by Abbott's formula (Abbott 1925). To determine differences among treatments in terms of mortality, the data were subjected to the analysis of variance (ANOVA) followed by LSD multiple comparison test. Statistical analyses were performed by SPSS 15.0 software.

Results

We determined the number of bacteria for per nymph by counting the number of colonies on the plates, which were inoculated with the diluted bacterial suspensions. The total number of bacteria was found to be 5.5×10^6 bacteria/nymph ($n = 50$).

A total of six bacteria were isolated from *T. aurantii* nymphs and they were identified based on their morphological, physiological, biochemical and molecular characteristics. Four bacteria were identified at species level, and the rest was identified at genus level. Colonies of five strains were cream on nutrient agar, and one isolate was orange. All colonies were smooth, except for Cb1, which was round-mucoid. Four isolates were bacil, and the rest was coccus. While the isolates of Cb1, Cb5 and Cb6 were gram-positive, and the other isolates were gram-negative. Only one isolate (Cb1) formed spore, which was central. None of them produced capsule, and only Cb1 was motile. All isolates caused turbidity in nutrient broth (Table 1).

Although all isolates were able to grow in nutrient broth medium containing 3% NaCl, only one isolate (Cb5) was able to grow in nutrient broth containing 12 and 15% NaCl. Growing at other NaCl concentrations was varied depending on the isolates. No isolates were grown in pH 3.0, 4.0, 10.0, and 12.0, and all of them were able grow in pH 6.0, 7.0, and 8.0. Growing at different temperature was varied depending on the isolates (Table 2).

All isolates were catalase-positive, and all were coagulase-negative. The other biochemical properties of the bacterial isolates are given in Table 3.

We also determined the partial sequence of the 16S rRNA gene for further characterization of the bacterial isolates. Approximately 1,150 bp fragment of the 16S rRNA gene region was sequenced. Based on all identification studies, the isolates were identified as *Bacillus tequilensis* Cb1, *Chryseobacterium stagni* Cb2, *Pseudomonas flourescens* Cb3, *Rahnella aquatilis* Cb4, *Staphylococcus* sp. Cb5 and *Staphylococcus* sp. Cb6 (Table 4). This identification was also supported by a dendrogram which was constructed using 16S rRNA gene sequences (Fig. 1). The 16S rRNA nucleotide sequences of the bacterial isolates were deposited in the GenBank nucleotide sequence database (Benson et al. 2012) under the following accession numbers: *Bacillus tequilensis* Cb1 (JN706759), *Chryseobacterium stagni* Cb2 (JN706760), *Pseudomonas flourescens* Cb3 (JN706761), *Rahnella aquatilis* Cb4

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

Growth/isolate number	Cb1	Cb2	Cb3	Cb4	Cb5	Cb6
3% NaCI	+	+	+	+	+	+
5% NaCI	+	–	+	+	+	+
7% NaCI	+	–	–	+	+	+
10% NaCI	–	–	–	–	+	+
12% NaCI	–	–	–	–	+	–
15% NaCI	–	–	–	–	+	–
pH 3	–	–	–	–	–	–
pH 4	–	–	–	–	–	–
pH 5	+	+	+	+	–	+
pH 6	+	+	+	+	+	+
pH 7	+	+	+	+	+	+
pH 8	+	+	+	+	+	+
pH 9	+	–	+	+	+	–
pH 10	–	–	–	–	–	–
pH 12	–	–	–	–	–	–
10 °C	–	–	WP[b]	WP	–	–
15 °C	–	–	+	+	–	–
30 °C	+	+	+	+	+	+
37 °C	+	+	+	+	+	+
45 °C	+	–	+	–	+	–
50 °C	WP	–	–	–	–	–
55 °C	–	–	–	–	–	–

[a] All tests were performed in Luria-Bertani broth.
[b] WP means weak positive.

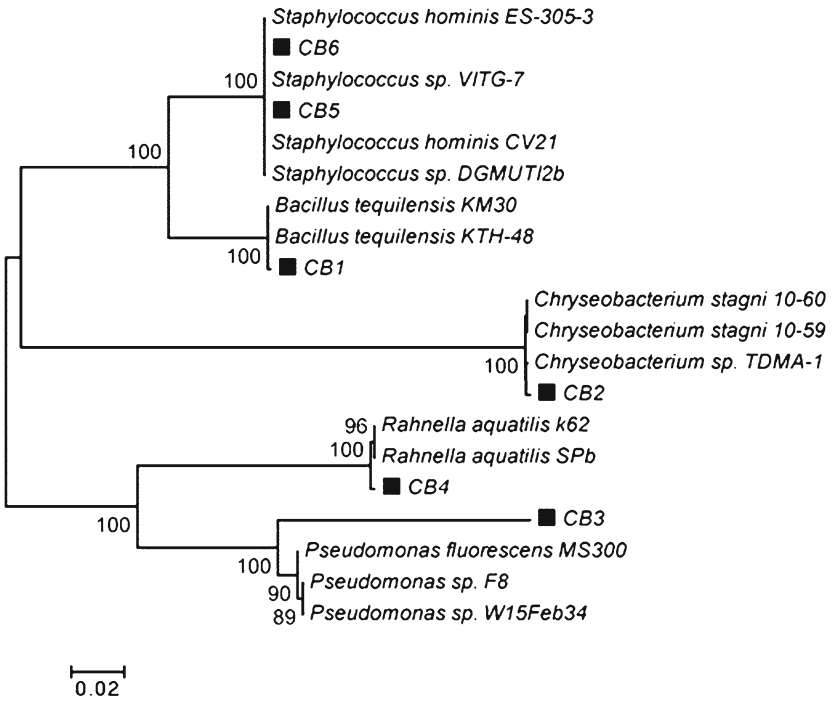

Fig. 1. Pyhlogenetic analysis of the bacterial isolates from *T. aurantii* and their closely related 14 bacterial species based on the partial sequence of the 16S rRNA gene. Neighbor-joining analysis with p-distance method was used to construct the dendrogram. Bootstrap values shown next to nodes are based on 1,000 replicates. Bootstrap values C $\geq$ 70% are labeled. *T. aurantii* isolates were indicated with black squares. The scale on the bottom of the dendrogram shows the degree of dissimilarity.

(JN706762), *Staphylococcus* sp. Cb5 (JN706763) and *Staphylococcus* sp. Cb6 (JN706764).

In addition, we tested the bacterial isolates against the last instar nymphs of the pest as a possible biological control agent. The highest mortality was obtained from *P. flourescens* Cb3 with 50% mortality within 10 days after application of the bacterial suspension ($F = 10.53$, $df = 6$, $p < 0.05$). The isolates of Cb1, Cb4, and Cb5 produced 10, 20, and 10% mortalities, respectively. However, these were not signifi-

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

Source	Isolate number					
	Cb1	Cb2	Cb3	Cb4	Cb5	Cb6
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	–	–	–
Nitrate reduction	+	–	–	+	+	+
Hydrolysis of starch	–	–	–	–	–	–
Hydrolysis of urea	–	–	–	–	+	+
Methyl red test	–	–	–	–	+	+
Voges Proskauer	–	–	–	–	–	–
Citrate	–	–	–	–	–	–
Gelatin hydrolysis	+	+	+	–	–	+
Coagulase	–	–	–	–	–	–
Kligler iron agar	Basic	Basic	Basic	Acid	Acid	Basic
Glycerol	–	–	–	+	WP[b]	WP
Erythritol	–	–	–	WP	–	–
D-arabinose	–	–	–	WP	–	–
L-arabinose	–	–	–	+	–	–
D-ribose	–	–	–	+	WP	–
D-xylose	–	–	–	+	–	–
L-xylose	–	–	–	–	–	–
D-adonitol	–	–	–	–	–	–
Methyl-β-D-xylopyranoside	–	–	–	–	–	–
D-galactose	–	–	–	+	+	+
D-glucose	+	–	–	+	+	+
D-fructose	+	–	–	+	+	+
D-mannose	+	–	–	+	+	–
D-sorbose	–	–	–	+	–	–
L-rhamnose	–	–	–	+	–	–
Dulcitol	–	–	–	+	–	–
Inositol	–	–	–	–	–	–
D-mannitol	–	–	–	+	+	–
D-sorbitol	–	–	–	–	–	–
Methyl-α-D-mannopyranoside	–	–	–	–	–	–
Methyl-α-D-glucopyranoside	–	–	–	+	+	–
N-acetylglucosamine	–	–	–	+	+	–
Amygdalin	–	–	–	WP	–	–
Arbutin	+	–	–	+	+	–
Esculin/ferric citrate	+	+	–	+	+	–
Salicin	+	–	–	+	+	–
D-cellobiose	+	–	–	+	+	–
D-maltose	–	–	–	+	+	+
D-lactose (bovine origin)	–	–	–	+	+	+
D-melibiose	–	–	–	+	+	–
D-sucrose	+	–	–	+	+	+
D-trehalose	+	–	–	+	+	–
Inulin	–	–	–	–	–	–
D-melezitose	–	–	–	–	WP	+
D-raffinose	–	–	–	+	+	–
Starch	–	–	–	+	–	–
Glycogen	–	–	–	–	–	–
Xylitol	–	–	–	–	–	–
Gentiobiose	–	–	–	WP	+	–
D-turanose	–	–	–	WP	WP	+
D-lyxose	–	–	–	–	–	–
D-tagatose	–	–	–	–	–	–
D-fucose	–	–	–	–	–	–
L-fucose	–	–	–	–	–	–
D-arabitol	–	–	–	–	–	–
L-arabitol	–	–	–	–	–	–
Gluconate	–	–	–	WP	–	–
2–Ketogluconate	–	–	–	WP	–	–
5–Ketogluconate	–	–	–	–	–	–

[a] The 1st through 11th tests were conventional; the rest were performed with API 50 CH.
[b] WP means weak positive.

cantly higher than the control (p>0.05). Cb2 and Cb6 caused the same mortality with the control ($p > 0.05$) (Fig. 2).

Discussion

There is a critical need to search for safe and effective alternatives to chemical insecticides, which are known

Table 4. Identification results of the bacterial isolates according to the partial sequence of the 16S rRNA gene region.

Isolate	Acc. Nos.	Suggestions of GenBank	Similarity (%)	Identities
Cb1	JF411311	*Bacillus tequilensis* KM30	99	1351/1363
	HM854243	*Bacillus tequilensis* KTH-48	99	1353/1366
Cb2	DQ314741	*Chryseobacterium stagni* 10–60	98	1334/1354
	DQ314742	*Chryseobacterium stagni* 10–59	98	1333/1354
	AB264124	*Chryseobacterium* sp. TDMA-1	98	1321/1340
Cb3	GU084125	*Pseudomonas* sp. F8	98	1337/1364
	HQ589333	*Pseudomonas fluorescens* MS300	98	1336/1363
	EU681017	*Pseudomonas* sp. W15Feb34	98	1336/1363
Cb4	AY253920	*Rahnella aquatilis* k 62	98	1344/1364
	FJ405361	*Rahnella aquatilis* SPb	98	1342/1364
Cb5	JF923460	*Staphylococcus* sp. DGM UTI2b	98	1362/1379
	AJ717375	*Staphylococcus hominis* CV21	98	1362/1379
Cb6	EU807748	*Staphylococcus* sp. VITG-7	99	1355/1363
	FN393804	*Staphylococcus hominis* ES-305–3	99	1359/1369

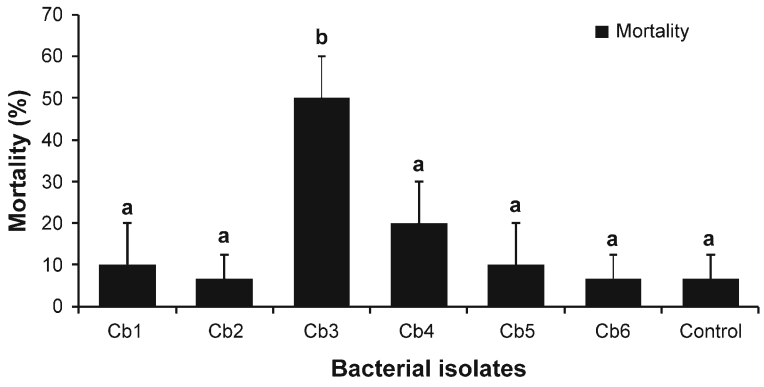

Fig. 2. Pathogenicity of the bacterial isolates against the last instar nymphs of *T. aurantii* within 10 days after application. Mortality data were corrected based on Abbott's formula (Abbott 1925). Different letters show the difference between isolates according to the LSD multiple comparison test ($p < 0.05$). Bars show standard deviation. Cb1: *Bacillus tequilensis*, Cb2: *Chryseobacterium stagni*, Cb3: *Pseudomonas flourescens*, Cb4: *Rahnella aquatilis*, Cb5: *Staphylococcus* sp., Cb6: *Staphylococcus* sp., Control: phosphate-buffered saline.

to show long persistence in the environment and leave toxic residues that affect human, plants and beneficial insects. This critical need has stimulated considerable interest in using insect pathogens as biological control agents. Microbial pesticides are becoming recognized as an important factor in crop and forest protection and in insect vector to provide sustainable and ecologically acceptable pest control strategies. In this study, we determined the culturable bacterial species associated with *T. aurantii* and their biocontrol potential. Six bacterial species including *B. tequilensis* Cb1, *C. stagni* Cb2, *P. flourescens* Cb3, *R. aquatilis* Cb4, *Staphylococcus* sp. Cb5 and Cb6 were isolated and characterized. The species detected in the present study were within the expected bacterial species based on previous studies performed on different insect species (Sezen et al. 2004; Bahar & Demirbag 2007; Lacey et al. 2007; Ince et al. 2008; Sevim et al. 2010; Muratoglu et al. 2011).

The highest insecticidal effect was recorded from *P. flourescens* Cb3 with 50% mortality value within 10 days after inoculation. *P. fluorescens* is a common Gram-negative, rod-shaped bacterium and it has biocontrol properties, protecting the roots of some plant species against parasitic fungi, such as *Fusarium* or *Pythium*, as well as some phytophagous nematodes (Palleroni 1984; Haas & Keel 2003). It has also been recognized as facultative insect pathogens (Krieg 1961). Sezen et al. (2004) isolated *P. flourescens* from alder leaf beetle (*Agalastica alni* (Coleoptera: Chrysomelidae)) and they showed that this bacterium caused 70% and 56% mortality against larvae and adult of the pest, respectively. Lipa & Wiland (1972) observed the highest insecticidal activity on isolate *P. fluorescens* strain 94-83a with 88% mortality by injection of bacterial suspension against *Agrotis segetum* (Lepidoptera: Noctuidae) larvae. Padmanabhan et al. (2005) showed that the formulation prepared from the exotoxin of *P. fluorescens* was toxic to the house fly. Inglis et al. (2000) determined that *Pseudomonas* spp. isolates were pathogenic to south-western corn borer (*Diatraea*

grandiosella (Lepidoptera: Crambidae)) larvae under controlled laboratory conditions. In this study, we isolated *P. flourescens* strain Cb3, which exhibited a significant mortality value against *T. aurantii*. Based on the infectivity test, this isolate appears to be a good candidate for further investigation as a possible biocontrol agent.

B. tequilensis is rod-shaped bacterium that is Gram-positive, motile, catalase- and oxidase-positive. This species was first isolated a shaft-tomb, and was shown to be distinct species within *Bacillus* genus, which is highly related to members of the*B. subtilis* subgroup (Gatson et al. 2006). Until now, there is no certain evidence that this bacterium is associated with insects. However, according to our knowledge, this is the first report indicating the isolation of *B. tequilensis* from any insect species; *T. aurantii* in this case. On the other hand, we could not observe good insecticidal activity from this bacterium against the *T. aurantii* nymphs, suggesting *B. tequilensis* may not be potentially an insect pathogen. However, further bioassay studies are needed to prove this.

C. stagni was isolated from any insect species with this study for the first time, although there are some studies showing the isolation of *Chryseobacterium* species from different insect species (Orborn et al. 2002; Lacey et al. 2007; Kampfer et al. 2010). It has been known that bacteria belonging to*Chryseobacterium* genus are not insect pathogen (Bernardet et al. 2006). Similarly, our results demonstrated that *C. stagni* Cb2 caused the same mortality as the control group against *T. aurantii*, indicating this bacterium is not an insect pathogen.

We isolated two *Staphylococcus* species (Cb7 and Cb8) from *T. aurantii* nymphs. But, these isolates did not exhibit good mortality against the pest. Bucher (1981) indicated that *Staphylococcus* species were rarely associated with insects. However, Kuzina et al. (2001) isolated three *Staphylococcus* species (*S. aureus*, *S. carnosus* and *S. xylosus*) from Mexican fruit flies *Anastrepha ludens* (Diptera: Tephritidae). Osborn et al. (2002) found three different *Staphylococcus* species (*S. gallinarum*, *S. sciuri* and *S. warneri*) from *Hylesia metabus* (Lepidoptera: Saturniidae). Ince et al. (2008) also isolated two *Staphylococcus* sp. strains from *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae) larvae. Moreover, Yu et al. (2008) isolated *Staphylococcus kloosii* from *Hepialus gonggaensis* (Lepidoptera: Hepialidae). All these studies suggest that *Staphylococcus* species could be closely related with insects. But it seems that they are not entomopathogenic.

R. aquatilis, a rare enteric gram-negative rod, has been widely recognized in environmental samples including fresh water, minced meat, freshwater fish, dairy products, and lager beer breweries (Harrell et al. 1989; Hamze et al. 1991; Lindberg et al. 1998). This bacterium has also clinical importance (Tash 2005). Gavini et al. (1976) first described this bacterium in 11 fresh water samples using numerical taxonomy and phenotypic characteristics. In addition, *R. aquatilis* has been isolated from different insect species with many studies, but the exact role of this organism in insect gut remains unknown (Lacey et al. 2007; Yu et al. 2008). In this study, we also isolated *R. aquatilis* from *T. aurantii*, however, it has no pathogenic effect towards the pest.

In conclusion, we isolated the culturable bacteria from *T. aurantii* collected from the Eastern Black Sea Region of Turkey, and characterized them in detail using a number of currently used techniques. This is an important step to investigate and understand the roles that such bacteria play in *T. aurantii* physiological development as well as their role in the natural regulation of the insect. We also tested these bacteria against the last instar nymphs of the pest. Among the bacteria tested, *Pseudomonas flourescens* Cb3 in particular may be a good candidate for further investigation as a possible biocontrol agent. However, further studies should include more detailed bioassay studies to explain certain biocontrol potential of the isolate Cb3. Field trials and transmission studies between adults and nymphs are also warranted. Moreover, general environmental risk assessments, such as toxicity tests against beneficial insects and mammals should also be done.

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