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Culturable bacterial microbiota of *Plagiodera versicolora* (L.) (Coleoptera: Chrysomelidae) and virulence of the isolated strains

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Abstract *Plagiodera versicolora* (Laicharting, 1781) (Coleoptera: Chrysomelidae) is an important forest pest which damages many trees such as willow, poplar, and hazelnut. In order to find new microbes that can be utilized as a possible microbial control agent against this pest, we investigated the culturable bacterial flora of it and tested the isolated bacteria against *P. versicolora* larvae and adults. We were able to isolate nine bacteria from larvae and adults. The isolates were characterized using a combination of morphological, biochemical, and physiological methods. Additionally, we sequenced the partial sequence of the 16S rRNA gene to verify conventional identification results. Based on characterization studies, the isolates were identified as *Staphylococcus* sp. Pv1, *Rahnella* sp. Pv2, *Rahnella* sp. Pv3, *Rahnella* sp. Pv4, *Rahnella* sp. Pv5, *Pantoea agglomerans* Pv6, *Staphylococcus* sp. Pv7, *Micrococcus luteus* Pv8, and *Rahnella* sp. Pv9. The highest insecticidal activity against larvae and adults was obtained from *M. luteus* Pv8 with 50 and 40 % mortalities within 10 days after treatment, respectively. Extracellular enzyme activity of the bacterial isolates such as amylase, proteinase, lipase, cellulose, and chitinase was also determined. Consequently, our results show that *M. luteus* Pv8 might be a good candidate as a possible microbial control agent against *P. versicolora* and were discussed with respect to biocontrol potential of the bacterial isolates.

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Introduction

The willow leaf beetle, *Plagiodera versicolora* (Laicharting, 1781) (Coleoptera: Chrysomelidae), is one of the most damaging pest species of various willow, poplar, birch, and hazelnut trees worldwide (Aslan 2001; Toros 1996). This pest occurs on large areas including northern Africa, America, Europe, and Asia (Urban 2005; Aslan and Ozbek 1999; Ishiara and Ohgushi 2006). It has also a wide distribution throughout Turkey (Çanakçıoğlu and Mol 1998). *P. versicolora* larvae and adults both feed on leaves of willows and other trees during the summer months. Adults chew holes in leaves or notches at leaf margins, and prefer new leaves. Larvae feed in groups or rows preferring older leaves. The larvae skeletonize the leaves, feeding on both sides of the leaves and eating the tissue between the veins. All the leaves may turn brown with a heavy infestation (Çanakçıoğlu and Mol 1998, 2000). Two or three generations may occur in a year depending on moisture and temperature (Aslan 2001).

So far, some control methods such as beetle collection with a broom, shaking off beetles into vessels, and burning of falling leaves including wintering beetles have been applied to reduce damaging effect of this pest (Urban 2005; Allegro 1989). However, these control methods were found

to be not effective. In addition, some chemical insecticides such as acephate, imidacloprid diflubenzuron, and carbofuran have been also utilized for controlling this pest (Jodal 1985; Cavalcaselle 1972; Allegro 1989). However, chemical insecticides have undesirable and hazardous side effect to the environment, humans, and plants. Also, many insect species have gained resistance against these chemical insecticides. Although some natural enemies of *P. versicolora* such as *Orthotylus marginalis*, *Anthocoris nemorum*, and *Harmonia axyridis* have been detected (Björkman et al. 2003), they do not offer control and are not currently used in any biocontrol program. Finally, as a microbial control agent, only the potential use of two B*acillus thuringiensis* isolates against *P. versicolora* has been investigated (Bauer 1992; Feng et al. 2000). There is still a need to find new microbes that can be used to control the willow leaf beetle.

Microbial pesticides are becoming recognized as an important factor in crop and forest protection. These pesticides are natural, disease-causing microorganisms such as viruses, bacteria, nematodes, protozoa, and fungi, which infect or intoxicate specific pest groups (Khetan 2001). Entomopathogenic bacteria mainly occur in the families of Bacillaceae, Pseudomonadaceae, Enterobacteriaceae, Streptococcaceae, and Micrococcaceae (Tanada and Kaya 1993; Garczynski and Siegel 2007). Today, the principal microbial insecticides utilize spore-forming bacteria (Bacillaceae) or toxins produced by these bacteria as their active ingredient, either in formulations or by incorporation of toxin genes with insecticidal properties into transgenic plants (Garczynski and Siegel 2007). Among spore-forming bacteria, the insecticidal bacterium *Bacillus thuringiensis* (Bt) has been the most successful and holds considerable potential for further development (Khetan 2001; Sevim et al. 2012a).

Insects are surrounded by a variety of microbes, and they should harbor some of them in the gut (Ishikawa 2003). The gut microbiota of insects represents all aspects of microbial relationships, from pathogenic to obligate mutualism (Dillon and Dillon 2004). Based on recent studies, it has been realized that symbiotic bacteria can be used as microbial control agents against insect pests in a few different ways. One of these approaches is that symbiotic bacteria can be genetically modified to express insecticidal toxins or detrimental substances (Beard et al. 1998). Medina et al. (2009) showed the successful transformation of the pZeoDsRed shuttle vector into fire ant midgut bacteria and suggested that the transformed bacteria can be served for the expression of toxic proteins in the gut. Moreover, Beard et al. (1992) found that the symbiotic bacteria of Chagas disease vector, *Rhodnius prolixus*, were transformed to express an anti-trypanosomal agent in the gut. This approach forms the basis of principle to utilize bacterial symbionts as a microbial control agent. Additionally, dynamics among bacterial microbes in the insect gut flora provide another interesting theme from a microecological point of view. Harada and Ishikawa (1997) showed that each species of bacteria from the aphids gut can be cultured and returned to the host by mixing the cells with a diet that the host ingests. Consequently, every species of the gut bacteria, when put back into aseptic aphids by itself, tended to proliferate excessively and eventually kill the hosts. This suggests that the microbes are potentially harmful to the host and can be compatible with it only when they form a multispecies community (Ishikawa 2003). Up to now, many scientists determined bacterial flora of many pest species in both agriculture and forestry (Danismazoglu et al. 2012; Secil et al. 2012; Yaman et al. 2010; Sevim et al. 2010; Ince et al. 2008; Gokce et al. 2010; Mrazek et al. 2008; Sevim et al. 2012b, c).

In the present study, we focused on the determination of the bacterial flora of the willow leaf beetle to find new microbes which can be utilized against it, and the identification of suitable bacterial isolates that could be candidate to express insect-killing proteins to provide new approaches of microbial control of *P. versicolora*. The isolated bacteria were identified using a number of currently used morphological, biochemical, physiological, and molecular techniques. Also, the insecticidal activity of the bacterial isolates was tested against *P. versicolora* larvae and adults under controlled laboratory conditions. This is the first study to determine the bacterial flora of *P. versicolora* and their pathogenicity towards to the pest.

Materials and methods

Collection of insects

P. versicolora larvae and adults were collected from the infested *Salix* spp. forests in the vicinity of Trabzon, Turkey during the summer months of 2011. Collected specimens were taken into plastic boxes (20 mm) with ventilated lids to allow airflow and were immediately transported to the laboratory. Insect samples were fed by freshly collected *Salix* spp. leaves at room temperature until bacterial isolation was performed.

Isolation of bacteria

The bacterial isolation was performed on both larvae and adults of *P. versicolora*. Ten insect specimens (ten larvae and ten adults) were separately surface sterilized with 70 % ethanol for 3 min by gently swirling and then washed two to three times with sterile distilled water (Lipa and Wiland 1972). The surface sterilized insects were placed in test tubes including 2 mL nutrient broth (Difco) and homogenized using a glass tissue grinder. After that, the

homogenates were filtered into sterile test tubes through two layers of sterile muslin to remove insect debris. The homogenates were diluted from 10^{-1} to 10^{-6}, and 10, 50, and 100 μL from these dilutions were spread on nutrient agar plates. Additionally, the remaining insect homogenates were heated at 80 °C to eliminate non-spore-forming bacteria, and then, 10, 50, and 100 μL from the heated suspensions were spread on nutrient agar plates. Plates were incubated at 30 °C for 2–3 days. At the end of the incubation period, bacterial colonies were separated based on their color and colony morphology. The discrete bacterial colonies were transferred to another nutrient agar plate and incubated for 48–72 h for growing. Purified bacterial isolates were subcultured and stored in 20 % glycerol at −80 °C for further characterization studies. The isolated bacterial strains were identified by a number of morphological, biochemical, physiological, and molecular techniques according to *Bergey's Manual of Systematic Bacteriology*, volumes 1 and 2 (Krieg and Holt 1986; Sneath et al. 1986). All isolates identified in this study are public-accessible and were deposited at Microbiology Laboratory, Department of Biology at Recep Tayyip Erdoğan University, Rize, Turkey.

Characterization of the bacterial isolates

Phenotypic characterization

Colony morphology of the bacterial isolates was inspected on nutrient agar by direct observations or using a stereomicroscope. The shape of the bacterial cells was determined using a light microscope at ×1,000 magnification. Gram staining of the bacterial isolates was carried out based on the method of Claus (1992). Endospore staining was performed according to the method described by Prescott et al. (1996). Capsule staining was carried out by negative staining. The motility of isolates was determined using a semisolid medium (Soutourina et al. 2001).

Biochemical characterization

Conventional biochemical tests such as KIA, oxidase, catalase, and indol were performed according to the *Bergey's Manual of Systematic Bacteriology*, volumes 1 and 2 (Krieg and Holt 1986; Sneath et al. 1986). Separately, extracellular enzyme activity of the bacterial isolates such as amylase, proteinase, lipase, cellulose, and chitinase was also determined. Protease activity was determined on agar plates including skim milk (Yu et al. 2009). Lipase activity was screened on rhodamine B (Sigma) agar plates including olive oil (Kouker and Jaeger 1987). Starch hydrolysis test was used to detect amylase activity (Yu et al. 2009). Chitinase activity was determined according to the procedure of Sandallı et al. (2008). Cellulose activity was determined on nutrient agar plates which included carboxymethyl cellulose (0.5 %). After 2 days of incubation, cellulose activity was determined with Congo red staining (Yu et al. 2009; Teather and Wood 1982). Formation of clear zones around the colonies was assessed as an indication of enzyme activity. Physiological tests such as temperature, pH, and NaCl tolerance were also performed in LB broth to determine physiological properties of the isolates.

Antimicrobial susceptibility testing

The antimicrobial susceptibility tests were performed by the standard disk diffusion test according to the National Committee for Clinical Laboratory Standards (NCCL Standards 1997). Mueller Hinton Agar (Merck, Germany) was used as the growth medium. The following antibiotic disk (Oxoid, UK) were used: streptomycin (10 μg), norfloxacin (10 μg), optochin (5 μg), vancomycin (30 μg), methicillin (5 μg), oxacillin (1 μg), novobiocin (30 μg), gentamycin (10 μg), amoxicillin (25 μg), tetracycline (30 μg), rifamycin (30 μg), sulfamethoxazole (25 μg), kanamycin (30 μg), erythromycin (15 μg), neomycin (30 μg), cephalothin (30 μg), ciprofioxacin (5 μg), chloranphenicol (30 μg), ampicillin (10 μg), amikacin (30 μg), ceftazidime (30 μg), and ceftriaxone (30 μg).

16S rRNA gene sequencing

For PCR, genomic DNA was extracted according to the standard protocol described by Sambrook et al. (1989). DNA pellets were dissolved in 50 μL Tris–EDTA buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 8.0). Isolated DNA was stored at −20 °C until use.

The oligonucleotide primers of 27F (5′-AGAGTTTGA TCMTGGCTCAG-3′ as forward) and 1492L (5′-GGYTACCTTGTTACGACTT-3′) as reverse (Macrogen) were used to amplify 16S rRNA gene for each bacterial isolate. PCR reactions contained 5 μL 10× *Taq* DNA polymerase reaction buffer, 1.5 μL 10 mmol/L dNTP mix, 1.5 μL 10 pmol each of the opposing primers, 1 μL 5 U/μL of *Taq* DNA polymerase (Fermentas), 3 μL $MgCl_2$, 2 μL genomic DNA, and 34.5 μL dH_2O. The PCR was performed under the following conditions: 2 min initial denaturation at 94 °C; 35 cycles of denaturation (45 s at 94 °C), annealing (60 s at 55 °C), and extension (60 s at 72 °C); a final extension at 72 °C for 10 min. Finally, PCR products were analyzed by electrophoresis through 1 % agarose gels and then visualized under UV light by staining with ethidium bromide. The right PCR products were sent to Macrogen (The Netherlands) for sequencing. The primers pairs of 518F (5′-CCAGCAGCCGCGGTAATACG-3′) and 800R (5′-TACCAGGGTATCTAATCC-3′) were used for

sequencing (Macrogen). The obtained sequences were analyzed by BLAST searches using the NCBI GenBank database (Altschul et al. 1990; Benson et al. 2012). Finally, the sequences were used to construct phylogenetic tree to verify isolate identification.

Nucleotide sequence accession numbers

The GenBank database accession numbers for the 16S rRNA nucleotide sequences of the bacterial isolates are as follows: *Staphylococcus* sp. Pv1, JQ522974; *Rahnella* sp. Pv2, JQ522975; *Rahnella* sp. Pv3, JQ522976; *Rahnella* sp. Pv4, JQ522977; *Rahnella* sp. Pv5, JQ522978; *Pantoea agglomerans* Pv6, JQ522979; *Staphylococcus* sp. Pv7, JQ522980; *Micrococcus luteus* Pv8, JQ522981; and *Rahnella* sp. Pv9, JQ522982.

Experimental infection

The bacterial isolates stocked at −80 °C were initially streaked on nutrient agar plates to obtain single colony for each isolate. After that, single colonies were inoculated into nutrient broth medium using an inoculation loop and incubated at 30 °C for 18 h (48 h for slow-growing isolates). At the end of the incubation period, the bacterial density was measured at 600 nm absorbance, and then adjusted to 1.89 (1.8×10^9 cfu/mL) (Ben-Dov et al. 1995; Moar et al. 1995). Finally, 5 mL of these cultures was centrifuged at 4,000 rpm for 15 min. The pellet was resuspended in 5 mL of sterilize phosphate buffer solution (PBS) and used for bioassay. Daily prepared bacterial solutions were used in bioassays.

Insect samples were collected from the vicinity of Trabzon, Turkey, in August of 2011. Specimens were transported to the laboratory and fed by *Salix* spp. leaves at room temperature. Insect samples were waited for approximately 2–3 days so that they were acclimated to the laboratory conditions. After that, randomly selected *P. versicolora* larvae and adults were used in bioassays. Freshly collected *Salix* spp. leaves were firstly washed with sterile distilled water for two to three times and were inoculated by 1 mL of bacterial suspension prepared as described above for each isolate. The leaves of the control group were treated with sterile PBS. After that, the contaminated leaves were placed in a plastic box (20 mm) with ventilated lids to permit airflow. Finally, third instar larvae and adults were put into each box and allowed to feed on the leaves. Experiments were performed with ten adults and ten larvae per replicate, and all experiments were repeated on different occasions. The boxes were incubated at room temperature under 12:12 photoperiod. After 3 days, fresh untreated leaves were provided every 3 days for 10 days.

Data analysis

The nucleotide sequences of the 16S rRNA genes belonging to the bacterial isolates of *P. versicolora* were initially aligned with the sequences of the related bacterial species from the GenBank database using the multiple alignment program ClustalW packaged in BioEdit (Hall 1999; Thomson et al. 1994). Phylogenetic relationships were inferred by the neighbor-joining method with p-distance analysis (Saitou and Nei 1987). The strength of the internal branches from the resulting tree was statistically tested by bootstrap analysis from 1,000 bootstrap replications (Felsenstein 1985). Analyses were performed with MEGA 5.0 software (Tamura et al. 2011).

Mortality data were corrected by Abbott's formula (Abbott 1925). The data were subjected to analysis of variance followed by post hoc LSD multiple comparison tests to compare isolates with each other and the control in terms of mortality ($p<0.05$). Chi-square test was used to determine difference between larvae and adults with respect to mortality. Statistical analyses were performed using SPSS 16.0 statistical software.

Results

In total, nine bacteria (five from larvae and four from adults) were isolated from *P. versicolora* and identified based on a variety of different techniques. Colonies of four isolates (Pv1, Pv3, Pv7, and Pv9) were cream on nutrient agar. Four isolates (Pv2, Pv4, Pv5, and Pv8) were yellow and one isolate (Pv6) was orange. All isolates were smooth on nutrient agar. Three isolates (Pv1, Pv7, and Pv8) were coccus, while five isolates were bacil or coccobacil. Three isolates (Pv1, Pv7, and Pv8) were gram positive, and the rest was gram negative. None of them formed spore and capsule. Four isolates (Pv2, Pv3, Pv5, and Pv9) were motile. All isolates caused turbidity in LB broth (Table 1).

Although all isolates were catalase positive, only one isolate (Pv2) was oxidase positive. Likewise, all isolates were negative in terms of gelatin hydrolysis. Although some of the isolates produced different extracellular enzymes such as amylase, lipase, protease, and cellulose, none of them produced chitinase [Electronic supplementary material (ESM) Table 1]. Other biochemical properties of the bacterial isolates were listed in ESM Table 1.

While all isolates were able to grow in 3, 5, 7, and 10 % NaCl concentrations, only one isolate (Pv1) was grown in 15 % NaCl concentration. Although none of isolates were grown in pH 3.0, pH 10, and pH 12, they were variable with respect to growing in other pH values. Although all isolates were able to grow at 10, 15, 30, and 37 °C, none of them were grown at 55 °C (ESM Table 2). Antibiotic resistance

Table 1 The morphological characteristics of the bacterial isolates

Isolate number	Pv1	Pv2	Pv3	Pv4	Pv5	Pv6	Pv7	Pv8	Pv9
Colony color	Cream	Yellow	Cream	Yellow	Yellow	Orange	Cream	Yellow	Cream
Colony shape	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Shape of bacteria	Coccus	Coccobacil	Coccobacil	Coccobacil	Coccobacil	Bacil	Coccus	Coccus	Coccobacil
Gram stain	+	−	−	−	−	−	+	+	−
Spore stain	−	−	−	−	−	−	−	−	−
Capsule	−	−	−	−	−	−	−	−	−
Motility	−	+	+	−	+	−	−	−	+
Turbidity when grown in NB	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid
Source	Larvae	Larvae	Larvae	Larvae	Larvae	Adult	Adult	Adult	Adult

profiles of the isolates were varied depending on the isolates and antibiotics used (ESM Table 3).

An approximately 1,400-bp fragment of the 16S rRNA gene region was also sequenced for further characterization of the isolates and to construct a dendrogram using closely related bacterial species of *P. versicolora* isolates. According to the identification studies, the isolates were identified as *Staphylococcus* sp. Pv1, *Rahnella* sp. Pv2, *Rahnella* sp. Pv3, *Rahnella* sp. Pv4, *Rahnella* sp. Pv5, *P. agglomerans* Pv6, *Staphylococcus* sp. Pv7, *M. luteus* Pv8, and *Rahnella* sp. Pv9 (Table 2). This identification was also supported by the phylogenetic analysis (Fig. 1).

Bacterial isolates caused different mortality values against *P. versicolora* larvae (F=10.27, df=9, p<0.05). The highest mortality was obtained from *M. luteus* Pv8 with 50 % within 10 days after treatment (F=10.27, df=9, p< 0.05). The other mortalities ranged from 0 to 13 % (Fig. 2). In the case of adult mortality, all isolates produced different mortalities in comparison to each other (F=3.34, df=9, p< 0.05). The highest mortality was obtained from *M. luteus* Pv8 with 40 % against *P. versicolora* adults (F=3.34, df=9, p<0.05). The other mortalities ranged from 0 to 13 % within 10 days after inoculation (Fig. 2). There was no significant difference between larval and adult mortality (p>0.05).

Discussion

A great number of microbial pest control products for controlling insect pests in both agriculture and forestry have been developed in the past and are developed at present because they offer theoretically one of the most sustainable and ecologically acceptable ways of crop and forest protection. Generally, they are intended to replace synthetic chemical pesticides as our society requires sustainable pest control solutions that are safe to the environment, humans, and plants. Therefore, research on new biocontrol agents which promise elegant solutions was frequently initiated by academic scientists. In this study, therefore, we investigated the bacterial flora of *P. versicolora* to find new microbes that can be used as possible microbial control agent against it and to find suitable symbiotic bacteria which could be candidate organism to express insecticidal toxins to provide new approaches of microbial control of the willow leaf beetle.

M. luteus is non-motile, nonspore-forming and strictly aerobic bacterium which produces yellow colonies on agar plates. Cells are gram-positive cocci arranged in tetrads. This bacterium could be found in soil, dust, water, air, and on human skin (Kocur et al. 1991). It has also been isolated from different insect species as a member of bacterial flora (Yılmaz et al. 2006; Yaman et al. 2002; Sezen et al. 2005; Lipa and Wiland 1972). It has also been shown that this bacterium has pathogenic effect against different insect species. Lipa and Wiland (1972) showed that *M. luteus* caused 8 % mortality against *Agrotis segetum* (Lepidoptera: Noctuidae) larvae. Yaman and Demirbag (2000) showed that this bacterium was pathogenic to *Pieris brassicae* (Lepidoptera: Pieridae) larvae. Sezen and Demirbag (1999) showed that *M. luteus* caused 30 % mortality against *Balanicus nucum* (Coleoptera: Curculionidae) larvae under laboratory conditions. Sezen and Demirbag (2006) demonstrated that *M. luteus* As4 caused 40 % mortality against *Agelastica alni* (Coleoptera: Chrysomelidae) larvae which is another important forest pest. Sezen et al. 2005 showed that *M. luteus* As4 caused 30 % mortality against *Amphimallon solstitiale* (Coleoptera: Scarabaeidae) larvae under laboratory conditions. In this study, we also showed that *M. luteus* Pv8 caused 50 and 40 % mortality against larvae and adults of *P. versicolora*, respectively. All these studies suggest that some strains of *M. luteus* seem to be insect pathogen. However, further studies such as calculation of LT_{50} and LC_{50} values are needed to prove this.

There are many studies showing the isolation of *Staphylococcus* species from different insect species. Sevim et al. (2012a) isolated two *Staphylococcus* strains

Table 2 Proposed identification of the bacterial isolates according to the BLAST search using the partial sequence of 16S rRNA gene

Isolate	Species	GenBank ID number	Query coverage (%)	Identity (%)
Pv1	*Staphylococcus hominis* CV21	AJ717375	100	99
	Staphylococcus sp. TPL10	EU373380	100	99
	Staphylococcus sp. HNR14	EU373372	100	99
Pv2	*Rahnella* sp. R707	GU299866	100	99
	Rahnella sp. CDC 1–576	U88434	100	99
	Rahnella aquatilis 3–88	X79940	100	99
Pv3	*Rahnella* sp. R707	GU299866	100	99
	Rahnella sp. WMR15	AM167519	99	99
	Rahnella sp. CDC 1–576	U88434	100	99
Pv4	*Ewingella americana* 48–2	AB675633	100	99
	Rahnella sp. CDC 1–576	U88434	100	99
	Rahnella sp. WMR15	AM167519	99	99
Pv5	*Rahnella* sp. R707	GU299866	100	99
	Rahnella sp. CDC 1–576	U88434	100	99
	Rahnella.aquatilis 2–87	X79937	100	99
Pv6	*Pantoea agglomerans* EM102	FJ357816	100	99
	Pantoea agglomerans SB545	FJ357834	100	99
	Pantoea agglomerans CE21	JN084142	99	99
	Pantoea agglomerans CE1	JN084132	99	99
Pv7	*Staphylococcus warneri* G72	HQ407248	99	100
	Staphylococcus pasteuri LCR12	HQ259721	99	100
	Staphylococcus sp. P106	EU195954	99	100
	Staphylococcus sp. LMG-19417	AJ276810	99	100
Pv8	*Micrococcus luteus* CV44	AJ717369	100	99
	Micrococcus luteus INBI-1	EU438932	100	99
	Micrococcus luteus INBI-4	EU438935	100	99
Pv9	*Ewingella americana* 48–2	AB675633	100	99
	Rahnella sp. WMR15	AM167519	100	99
	Rahnella sp. CDC21234	U88435	100	99
	Rahnella.aquatilis 2–87	X79937	100	98
	Rahnella aquatilis SPb	FJ405361	100	98
	Ewingella americana GTC1277	AB273745	100	98

from the black citrus aphid (*Toxoptera aurantii* (Homoptera: Aphididae)). Ince et al. (2008) also isolated two *Staphylococcus* strains from the pine processionary caterpillar (*Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae)). Ozkan et al. (2012) showed the isolation of one *Staphylococcus* strain from *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae (unpublished). Moreover, *Staphylococcus aureus*, *Staphylococcus carnosus*, *Staphylococcus xylosus*, *Staphylococcus gallinarum*, *Staphylococcus sciuri*, *Staphylococcus warneri*, and *Staphylococcus kloosii* were also isolated from different insects belonging to different orders such as Diptera and Lepidoptera (Kuzina et al. 2001; Osborn et al. 2002; Yu et al. 2008). In this study, we also isolated two *Staphylococcus* species from *P. versicolora* larvae and adults. However, they did not cause significant mortality against *P. versicolora* larvae and adults compared to the control group. Based on all these studies, it is possible to say that *Staphylococcus* species could be closely associated with insects. But there is no certain evidence that *Staphylococcus* species are insect pathogen.

The genus *Rahnella* is a member of the family of Enterobacteriaceae. The members of this genus are commonly found in the environment including rhizosphere, phyllosphere, fruits, water, and intestinal tracts of herbivores and insects (Berge et al. 1991; Hashidoko et al. 2002; Lindow et al. 1998; Niemi et al. 2001; Brenner et al. 1998; Sevim et al. 2012a; Lacey et al. 2007). Species within this genus is rarely isolated from human clinical specimens of immunocompromised patients (Harrell et al. 1989). This genus includes three closely related species such as *Rahnella aquatilis*, *Rahnella genomospecies* 2, and

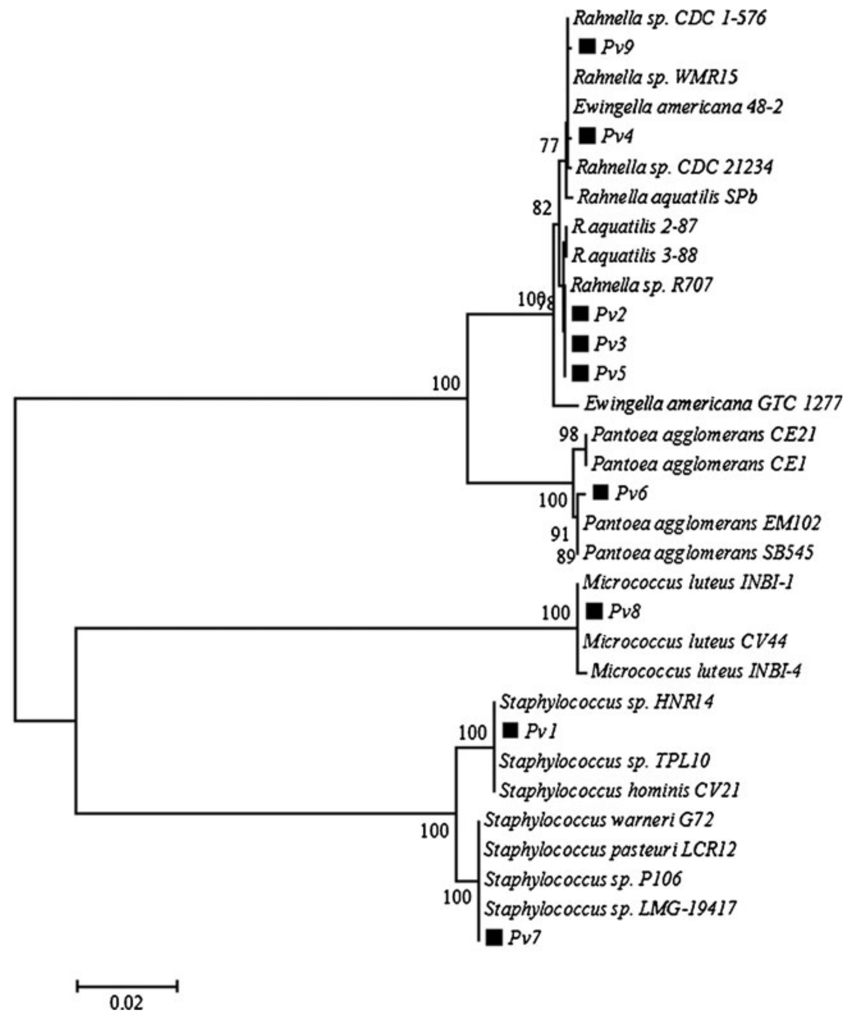

Fig. 1 The neighbor-joining tree of the bacterial isolates and their closely related bacterial species. The approximately 1,400-bp sequence of the 16S rRNA gene was used to construct the dendrogram. Bootstrap values based on 1,000 replicates were indicated *above* nodes. Bootstrap values C ≥70 are labeled. *P. versicolora* isolates were indicated with *black squares*. The scale on the bottom of the dendrogram indicates the degree of dissimilarity

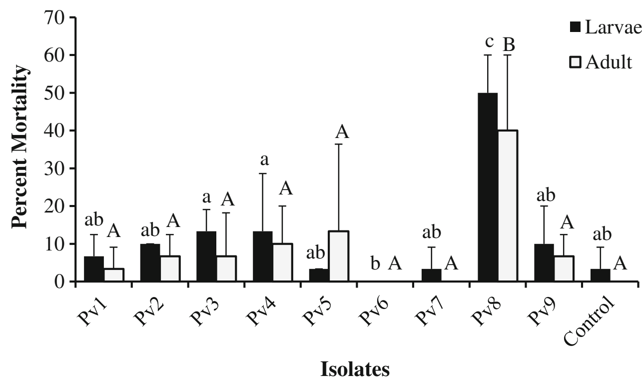

Fig. 2 Pathogenicity of the bacterial isolates using the bacterial concentration of 1.89×10^9 cfu/ml against *P. versicolora* larvae and adults within 10 days after application. Mortality data were corrected according to the Abbott's formula (Abbott 1925). *Bars* indicate standard deviation. *Different uppercase* and *lowercase letters* represent statistically significant differences among larval and adult mortalities. *Pv1 Staphylococcus* sp., *Pv2 Rahnella* sp., *Pv3 Rahnella* sp., *Pv4 Rahnella* sp., *Pv5 Rahnella* sp., *Pv6 P. Agglomerans*, *Pv7 Staphylococcus* sp., *Pv8 M. luteus*, *Pv9 Rahnella* sp.

Rahnella genomospecies 3, and these species cannot be phenotypically differentiated (Brenner et al. 1998). In the present study, we isolated five *Rahnella* species from *P. versicolora*, and they did not show good mortality against larvae and adults. All these studies suggest that *Rahnella* species are closely related with insects, but not pathogenic relation. The role of this genus in the insect gut is unknown, and further studies are needed to elucidate the function of these bacteria.

P. agglomerans (formerly known as *Enterobacter agglomerans*) is a gram-negative aerobic bacillus which is a member of the family Enterobacteriaceae. It is an environmental organism that is commonly isolated from plants, water, animal, and human feces (Andersson et al. 1999; Gavini et al. 1989). It has also been isolated from immunocompromised patients, causing human disease (Andrea et al. 2007). This bacterium has also been shown to be found in the gut of locusts, honey bees, bark beetles, and mosquitoes (Dillon et al. 2000; Loncaric et al. 2009; Bisi and Lampe 2011; Sevim et al. 2012b). In this study, we isolated *P. agglomerans* Pv6 from *P. versicolora* adults and showed

that this bacterium had no significant insecticidal activity against both larvae and adults of *P. versicolora*.

Symbiotic bacteria can be used to synthesize insect-killing proteins or toxins to provide new approaches of microbial control of insect pests. Schnepf and Whiteley (1981) showed that *Escherichia coli* strain Hb101 was successfully transformed to express *cry1Aa* gene, and they demonstrated that the recombinant bacterium showed the insecticidal activity similar to Cry1Aa synthesized in *B. thuringiensis*. Beard et al. (1992) demonstrated that the symbiotic bacteria of Chagas disease vector, *R. prolixus*, were genetically modified to express an anti-trypanosomal agent in the gut of Chagas disease vector. Moreover, Bisi and Lampe (2011) demonstrated that *P. agglomerans*, which is a bacterial symbiont of mosquitoes, was engineered to express anti-*Plasmodium* effector proteins such as anti-Pbs21 and PLA2, and they found that this bacterium successfully secreted these proteins. They also showed that the recombinant bacterium can grow efficiently like wild-type strain. In this study, we provided suitable bacterial strains that could be genetically modified to synthesize the insecticidal toxins for controlling the willow leaf beetle.

It has been hypothesized that the gut microorganisms may play a role in nutrition and digestion process of insects (Appel 1994; Anand et al. 2010). For instance, it has been shown that the gut bacteria of Lepidopteran insects produce digestive enzymes that help digestion of mulberry leaf constituents such as cellulose, xylan, pectin, and starch (Dillon and Dillon 2004). Moreover, Anand et al. (2010) found that bacteria in the gut of *Bombyx mori* can digest a number of polysaccharides such as cellulose, xylan, pectin, and starch, and they suggest that bacteria provide digestive enzymes in a synergic manner and contribute to larval growth. In this study, we also determined some of enzymes such as amylase, cellulose, lipase, protease, and chitinase produced by gut bacteria of *P. versicolora*. This should be an important step to understand the roles such bacteria play in *P. versicolora* physiological development and nutrition digestion.

In conclusion, we isolated and characterized the culturable bacterial flora of *P. versicolora* using a combination of currently used methods. We also tested the insecticidal activity of the bacterial isolates against both *P. versicolora* larvae and adults. Based on the pathogenicity test, *M. luteus* Pv8 might be a good candidate for further investigation as a possible microbial control agent. However, further studies are needed to determine the effectiveness of the bacterium in the field. Separately, the obtained bacterial strains from this study could be used to investigate the expression of the foreign genes to allow new approaches of the willow leaf beetle management. Moreover, this study could be a good guide to elucidate roles of the gut bacteria of the willow leaf beetle in the digestive process.

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