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ORIGINAL ARTICLE

Bacterial pathogens from *Diprion pini* L. (Hymenoptera: Diprionidae) and their biocontrol potential

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Abstract

Microbial insecticides have become increasingly important in recent years for the control of insect pests. Among microbial insecticides, insect pathogenic bacteria and their toxins have been the most commercially successful and hold potential for further development. In this study, we investigated the isolation and identification of some potential pathogenic bacteria from *Diprion pini* L. (Hymenoptera: Diprionidae) which is a serious pest of pine forests worldwide. A total of eighteen bacteria were isolated, ten bacteria from dead *D. pini* larvae and eight bacteria from healthy larvae. The bacterial strains were characterized by their morphological features, 16 S rRNA gene sequencing and phylogenetic analysis. In addition, these bacteria and four *Bacillus thuringiensis* strains (isolated from pine forest soil) were tested against *D. pini* larvae under laboratory conditions. The bacterial isolates were identified as *Kluyvera intermedia* O-1, O-8, O-10 and S-3, *Proteus mirabilis* O-2, *Klebsiella oxytoca* O-3, *Bacillus* sp. O-4, *Pantoea agglomerans* O-5 and S-5, *Serratia marcescens* O-6, *Pseudomonas* sp. O-7, *Acinetobacter* sp. O-9, *Enterobacter* sp. S-1 and S-9, *Bacillus pumilus* S-2, *Enterobacter cancerogenus* S-4, *Pseudoclavibacter* sp. S-6 and *Arthrobacter* sp. S-8. All isolates showed different insecticidal activity against the pest and the highest mortality was obtained from *P. mirabilis* O-2 with 100% within ten days after exposure. The highest mortality among *B. thuringiensis* strains was obtained from *B. thuringiensis* 37 – 4 with 56.67%. This is the first study of determination of the culturable bacterial diversity within *D. pini* and the obtained results might be beneficial for biocontrol of *D. pini*.

Keywords The common pine sawfly · Bacteria · Virulence · Biological control

Abbreviations

16 S rRNA	16 S ribosomal RNA.
ANOVA	Analysis of Variance.
BLAST	The Basic Local Alignment Search Tool.
Bt	*Bacillus thuringiensis*.
CA	California.
cfu	Colony-forming unit.
cry	crystal toxin.
DNA	Deoxyribonucleic acid.
dNTP	Deoxynucleotide triphosphates.
MA	Massachusetts.
MEGA	Molecular Evolutionary Genetics Analysis.
MD	Maryland.
NCBI	The National Center for Biotechnology Information.
N-J	Neighbor-joining.
LSD	Fisher's least significant difference.
OD	Optical density.
PBS	Phosphate buffer solution.
PCR	Polymerase chain reaction.
RNA	Ribonucleic acid.
USA	The United States of America.
UV	Ultraviolet.

Introduction

The common pine sawfly, [*Diprion pini* L. (Hymenoptera: Diprionidae)], is a species of sawfly in the genus of *Diprion.* This insect causes economic damage to pine forests almost

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everywhere in the world and can cause defoliating and the death of pine trees in large pine forests. The effected trees weaken in time as the pest feeds until the end of autumn and eventually die if they are not appropriately controlled (Knerer 1993; Sharov 1993; Augustaitis 2007; Pschorn-Walcher 1982; Herz and Heitland 2003). Larvae of the pest feed on needles of several pine species, especially yellow pine (*Pinus sylvestris* L.) (Meshkova et al. 2019). This pest can cause the death of pine trees, and in some cases even if it doesn't kill trees, the weakened trees become host and attacked by other pests such as bark beetles (Knerer 1993; Geri 1988; Långström et al. 2001). The pest's economic damage in forests is so great that it was reported that the volume growth decreases in the rate of 86% in moderate defoliation and 94% in severe defoliation, in addition to the decrease in reproductive activity and lumber yield in trees. During epidemics, the death of the host tree can occur, and the death rate can be up to 30% (Lyytikäinen-Saarenmaa and Tomppo 2002). In this sense, it is inevitable to control this pest by environment-friendly methods as an alternative to chemical insecticides when the economic losses caused by the pest are considered.

Up to now, different control methods have been used against *D. pini*. If the number of larvae in the infested trees is low, it is recommended to collect and destroy them within the scope of mechanical control. In addition, biological control can be applied by hanging bird nests in the forests and transporting the red wood ant (*Formica rufa* L. (Hymenoptera: Formicidae)) which is a well-known predator of the pest (Eroğlu 2017). There are also some studies regarding the control of *D. pini* using pheromone traps (Şimşek 2004). In case of major outbreaks and mass reproduction, contact, stomach poisons and chitin preventive insecticides in liquid and powder forms can be used but the usage of them is not recommended and subject to special permission at least in Turkey (Eroğlu 2017). All these methods used in the control of *D. pini* seem to be inadequate and costly, except for chemical control. However, considering the negative effects of chemical insecticides on the environment and human health, biological control methods have gained importance as an alternative. Many countries around the world prefer environment-friendly control methods against insect pests in both agriculture and forestry and scientists have been mostly worked on these areas.

Many species of animals including insects harbor symbiotic microorganisms inside their bodies. Insects, considered the largest group in the animal kingdom, have endosymbiotic relationships with bacteria ranging from obligate mutualism to facultative parasitism (Kikuchi 2009). Although insects have symbiotic relationships with many bacterial species, a limited number of bacterial species have pathogenic effects on insects (Ruiu 2015). These disease-causing bacteria in insects are called as entomopathogenic bacteria, and these and especially their toxins are the products of the most commercial importance among microbial pesticides (Glare et al. 2017). Among all entomopathogenic bacteria, *Bacillus thuringiensis* (*Bt*), is a gram positive and spore-forming bacterium, has a high economic value as a biocontrol agent (Ben-Dov 2014). This bacterium produces proteins called as delta-endotoxin or Cry proteins which show insecticidal activity in crystal form during the sporulation phase. Delta endotoxins are highly specific to target insects and environment-friendly (Bravo et al. 2007). These insecticidal proteins can be produced in sprayable spore/crystal formula or expressed in transgenic plants to control many harmful insects in the orders of Lepidoptera, Coleoptera and Diptera (Boncheva vd. 2006). Despite environmental and the host-plant factors, predators, pathogens and parasitoids are thought to be the important elements regulating and controlling *D. pini* populations especially during outbreaks (Stenberg 2015). Although many entomopathogenic bacteria have been identified from different insect species, soil, and other environments (Sezen et al. 2004; Yılmaz et al. 2006; Çelebi et al. 2014; Seçil et al. 2012; Gonzales et al. 2013), the isolation or testing of novel species or strains is still a desirable issue especially considering the ecological compatibility of indigenous bacterial strains with insect species or their environment.

Therefore, our study focused on isolation and characterization of possible pathogenic bacteria from healthy and dead *D. pini* larvae and testing them against the pest under laboratory conditions to determine their biocontrol potential. In addition, four different *B. thuringiensis* strains which were previously isolated from pine forest soils in the study region were tested against the pest since they are indigenous strains and might have a good potential as a biological control agent. The obtained results might be beneficial in the future control programs of the pest.

Materials and methods

Isolation of bacteria

The bacterial isolation was performed on the third or fourth instar *D. pini* larvae which were collected from yellow pine forests in Kırşehir, Turkey. The field collected larvae were brought to the laboratory in a paper bag with a small branch of yellow pine. After that, they were fed by pine needles for a couple of days in the laboratory and observed with respect to any diseased or dead larvae. Dead larvae showing any kind of symptoms were separated from healthy ones and the bacterial isolation was separately performed from healthy and dead larvae. A total of 10 healthy and dead larvae for

each was used for bacterial isolation. Ten healthy and dead larvae were separately surface sterilized with 70% ethanol to prevent possible surface contamination and then they were washed with sterile water twice. To prove the ethanol-based surface sterilization of larvae, 100 µl from the last washing water was plated on nutrient agar and incubated at 30 °C for two days. As a result, petri dishes showing no growth were considered successful in terms of surface sterilization. The surface-sterilized healthy and dead larvae were separately placed in sterile test tube containing 5 mL nutrient broth medium and homogenized by a handheld homogenizer for 5 min. After that, the larvae suspensions were filtered through two layer of sterile cheese cloth to remove insect debris and serial dilutions from the filtered suspensions were prepared from 10^{-1} to 10^{-6}. 100 µl from each dilution was plated on nutrient agar and incubated at 30 °C for two days in dark. After the incubation period, all bacterial colonies were carefully inspected and selected based on their morphology. The selected colonies were separately streaked on another nutrient agar and the resulting colonies were used for identification processes. All bacterial strains were stocked in 20% glycerol for further studies in Molecular Microbiology laboratory, Department of Plant Protection, Kırşehir Ahi Evran University and publicly accessible (Sevim et al. 2012, 2018; Demirci et al. 2013).

Phenotypic characterization

Bacterial colony morphologies were determined on nutrient agar after incubation at 30 °C for two days in dark using a stereo microscope. The cell shape of the bacterial strains was determined using a binocular microscope at 1000 × magnification. Gram staining, endospore staining, and the motility of the cells were determined according to the methods of Claus (1992), Prescott et al. (1996) and Soutourina et al. 2001, respectively. Negative staining was used to determine whether the bacterial isolates had capsules or not.

16S rRNA gene sequencing

Until now, the 16 S rRNA gene sequence has been the most common genetic marker to study bacterial phylogeny and used for species identification of many bacteria (Patel 2001; Janda and Abbott 2007). To perform 16 S rRNA gene sequencing, genomic DNAs from the bacterial isolates were extracted using PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, Invitrogen, Waltham, MA, USA) according to the manufacturer's recommendations. The extracted genomic DNAs were checked using agarose gel electrophoresis and stocked at -20 °C for further analysis.

16 S rRNA genes from the bacterial isolates were amplified by PCR using the amplification primers of 27 F (5′-AGAGTTTGATCMTGGCTCAG-3′ as forward) and 1492 L (5′- GGYTACCTTGTTACGACTT-3′) as reverse provided by MACROGEN (The Netherlands). The purified and quantified genomic DNAs was added to the amplification reaction mixture containing 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, 1.25 µL 5 U/µL of *Taq* DNA polymerase (Fermentas, MD, USA) and 3 µL $MgCl_2$. The final volume was completed to 50 µl by sterile ddH_2O. The reaction mixtures were incubated in a thermal cycler (Bio-Rad, CA, USA) as follows: 2 min initial denaturation at 95 °C; 35 cycles of denaturation (50 s at 94 °C), annealing (60 s at 55 °C), and extension (1.5 min s at 72 °C); a final extension at 72 °C for 10 min. Negative control (water as template) was provided for all PCRs. As soon as PCRs were over, 4 µl from each reaction were loaded on 1.2% agarose gel and run at 90 V for 45 min. After that, the gel was stained with ethidium bromide and then visualized under UV light. The 25 µL of PCR products were sent to MACROGEN (the Netherlands) for sequencing. The primer pairs 518 F (5′-CCAGC AGCCGCGGTAATACG-3′) and 800R (5′-TACCAGG GTATCTAATCC-3′) were used for sequencing (Sevim et al. 2018). The edited DNA sequences were used for the nucleotide BLAST searches in the NCBI GenBank database and the percent similarity of 16 S rRNA genes belonging to the bacterial isolates with the most related bacterial species was obtained (Altschul et al. 1990; Benson et al. 2012). ≥99% similarity of the 16 S rRNA gene sequences was chosen as a criterion to identify the isolates at species level, 97 to 99% similarity of the 16 S rRNA gene sequences was used to identify isolates at the genus level, and < 97% similarity of the 16 S rRNA gene sequences was used to define a potentially novel bacterial species (Drancourt et al. 2000, 2004).

Accession numbers or 16 S rRNA sequences

All 16 S rRNA gene sequences obtained from this study were deposited in GenBank under the accession numbers of MW719857-MW719873.

Biological infection experiments

All bacteria isolated from healthy and dead larvae of *D. pini* were tested against the third or fourth instar *D. pini* larvae under laboratory conditions. Additionally, we tested four *Bacillus thuringiensis* strains (M1-1, Y3-3, Y3-4 and 37 – 4) isolated from pine forest soils in Kırşehir, Turkey. To perform artificial infection experiments, all bacterial isolates from the cryopreserved cultures were firstly streaked on nutrient agar and incubated at 30 °C overnight. After the incubation, single colonies were selected for each isolate and separately

inoculated in 3 mL of nutrient broth and incubated at 30 °C overnight. After that, the bacterial density was determined at 600 nm absorbance and the optical density was adjusted to 1.89. This OD was estimated to be equal to 1.8×10^9 cfu/mL (Ben-Dov et al. 1995; Moar et al. 1995). The adjusted cultures were centrifuged at 5.000 rpm for 15 min and the supernatants were discharged. The remaining pellets were resuspended in 5 mL sterile PBS (phosphate buffer solution) buffer and directly used for experimental infection. The freshly prepared bacterial suspensions were used in infection experiments (Demirci et al. 2013).

D. pini larvae required for the infection experiments were collected from Kırşehir, Turkey from which yellow pine plantations and *D. pini* are very high populations and, put into plastic boxes (30 × 40 × 20 cm) with fresh pine needles as food. After that, they were transported to the laboratory and fed around two days to separate diseased or injured larvae. Finally, randomly selected healthy third-fourth instar *D. pini* larvae were used for bioassays. The daily collected yellow pine needles (around twenty needles for each replicate) were dipped into the bacterial suspensions as adjusted to $OD_{600} = 1.89$ before and made sure that all needles were contaminated with bacteria. Following this, the contaminated needles were put into small plastic boxes (20 × 10 × 10 cm) and ten third-fourth instar larvae were gently placed in these boxes for each replicate. All experiments were repeated three times on different occasions and time. All boxes were incubated at room temperature under 12:12 (L:D) photoperiod and daily inspected. After all contaminated needles were eaten by the larvae, the uncontaminated fresh needles were provided to the larvae as food. The needles of the control group were only dipped into sterile PBS. Finally, all boxes were daily checked with respect to dead larvae for ten days. For each day, dead larvae were counted for all boxes and the control group.

Data analysis

For Blast search and phylogenetic analysis, 16 S rRNA sequences of all bacterial isolates were edited by Bioedit software and approximately 1.400 bp sequences were obtained (Hall 1999). And then, all sequences belonging to the bacterial isolates and their most closely associated bacterial species were aligned using Clustal W packed in Bioedit. According to the alignment, all sequences were trimmed from the beginning and the end at the appropriate position to remove non-homologous sites. Finally, a phylogram was generated by neighbor-joining (N-J) analysis with p-distance correction using MEGA X software. Bootstrap analysis was performed with 1.000 pseudoreplicates to assess the confidence for each clade of the observed tree (Kumar et al. 2018).

Mortality data obtained from the infection experiments were calculated as percent data according to Abbott's formula (Abbott 1925). The significant differences among the bacterial isolates including *B. thuringiensis* isolates were determined by Analysis of Variance (ANOVA) and followed by the LSD multiple comparison test. To determine the difference between the bacterial isolates and the control group, data were subjected to ANOVA and afterwards Dunnett's one-tailed t-test. The bacterial isolates from healthy, dead larvae and *Bt* isolates were separately evaluated. All mortality data were tested for the homogeneity of variance using Levene's statistic and all percentage data were subjected to arcsin transformation. The normality of all data was assessed by Anderson-Darling test using Minitab 17 software. All other statistical tests were carried out using SPSS 16.0 software.

Results

A total of ten bacteria from dead larvae and eight bacteria from healthy larvae were isolated. Based on the morphological characterization studies, all bacteria from dead larvae had cream colony color, except for O-1 which was yellow. The colony color of bacteria from healthy larvae were cream (S-1, S-2, S-3, S-4, S-8, and S-9) and yellow (S-5 and S-6). Colony shape of the bacterial isolates were smooth, except for O-4 (from dead larvae) and S-2 (from healthy larvae). While only one strain (O-4) was Gram positive in dead larvae, four strains (S-2, S-6, S-8, and S-9) were Gram positive in healthy larvae. Only two strains (O-4 from dead and S-2 from healthy) included endospore. The other morphological properties of the isolates were given in Table 1.

In addition to morphological characterization, all bacterial isolates were identified by 16 S rRNA gene sequencing. The obtained 16 S rRNA gene sequences were subjected to Blast search and their percent similarity with their the most closely related bacterial species were revealed. This was used to confirm morphological identification (Table 2). Finally, phylogenetic analysis was performed to determine the exact identification of the isolates (Figs. 1 and 2). According to all characterization studies, the bacterial isolates were identified as *Kluyvera intermedia* O-1, O-8, O-10 and S-3, *Proteus mirabilis* O-2, *Klebsiella oxytoca* O-3, *Bacillus* sp. O-4, *Pantoea agglomerans* O-5 and S-5, *Serratia marcescens* O-6, *Pseudomonas* sp. O-7, *Acinetobacter* sp. O-9, *Enterobacter* sp. S-1 and S-9, *Bacillus pumilus* S-2, *Enterobacter cancerogenus* S-4, *Pseudoclavibacter* sp. S-6 and *Arthrobacter* sp. S-8.

We also tested all bacterial isolates and four *B. thuringiensis* isolates against the larvae of the pests. Bacterial isolates from dead, healthy larvae and *B. thuringiensis* isolates

Table 1 Morphological characteristics of the bacterial isolates from dead and healthy *D. pini* larvae

Isolates from dead larvae	Colony color	Colony shape	Shape of Bacteria	Gram Strain	Spore Strain	Place of Spore	Shape of Spore	Capsule	Motility	Turbidity*	Source
O-1	Yellow	Smooth	Bacil	-	-	-	-	-	+	Turbid	Larvae
O-2	Cream	Smooth	Bacil	-	-	-	-	-	+	Turbid	Larvae
O-3	Cream	Smooth	Bacil	-	-	-	-	-	+	Turbid	Larvae
O-4	Cream	Rough	Bacil	+	+	Central	Oval	-	+	Turbid	Larvae
O-5	Cream	Smooth	Bacil	-	-	-	-	-	+	Turbid	Larvae
O-6	Cream	Smooth	Coccus	-	-	-	-	-	+	Turbid	Larvae
O-7	Cream	Smooth	Bacil	-	-	-	-	-	+	Turbid	Larvae
O-8	Cream	Smooth	Coccus	-	-	-	-	-	-	Turbid	Larvae
O-9	Cream	Smooth	Coccus	-	-	-	-	-	+	Turbid	Larvae
O-10	Cream	Smooth	Bacil	-	-	-	-	-	+	Turbid	Larvae
Isolates from healthy larvae											
S-1	Cream	Smooth	Coccus	-	-	-	-	-	+	Turbid	Larvae
S-2	Cream	Rough	Bacil	+	+	Central	Cylindrical	-	+	Turbid	Larvae
S-3	Cream	Smooth	Coccus	-	-	-	-	-	+	Turbid	Larvae
S-4	Cream	Smooth	Bacil	-	-	-	-	-	+	Turbid	Larvae
S-5	Yellow	Smooth	Bacil	-	-	-	-	-	-	Turbid	Larvae
S-6	Yellow	Smooth	Bacil	+	-	-	-	-	+	Turbid	Larvae
S-8	Cream	Smooth	Bacil	+	-	-	-	-	+	Turbid	Larvae
S-9	Cream	Smooth	Coccobacil	+	-	-	-	-	+	Turbid	Larvae

* When grown in nutrient broth

were separately evaluated in statistical analysis. There was a significant difference among the bacterial isolates from dead larvae with respect to mortality ($F = 3.61$, $df = 9$, $p < 0.05$). The highest mortality was obtained from *P. mirabilis* O-2 with 100% mortality ($F = 3.61$, $df = 9$, $p < 0.05$). The control group was different from the bacterial isolates ($F = 5.09$, $df = 10$, $p < 0.05$) (Fig. 3). For the bacterial isolates from healthy larvae, there was a significant difference among the isolates with respect to mortality ($F = 8.75$, $df = 7$, $p < 0.05$). The highest mortality was obtained from *E. cancerogenus* S-4 with 86% ($F = 8.75$, $df = 7$, $p < 0.05$). The control group was different from all isolates from healthy larvae ($F = 9.72$, $df = 8$, $p < 0.05$) (Fig. 4). There was no significant difference among *B. thuringiensis* isolates in terms of mortality ($F = 1.04$, $df = 3$, $p > 0.05$). The control group wasn't different from *B. thuringiensis* isolates in terms of mortality ($F = 1.77$, $df = 4$, $p > 0.05$) (Fig. 5). All mortality values were provided on Figs. 3, 4 and 5.

Discussion

The negative effects of chemical insecticides used in agriculture and forestry on the environment, human, animal health and non-target organisms has long been a well-known issue. In addition, the commonly used pesticides can be detected in air, soil, water, and plants at different rates, which are the basic components of the environment, and even in all living tissues, especially animals and humans (Babayiğit et al. 2014). At the same time, the overuse of insecticides causes the development of insecticide resistance in insects, and as a result, pesticide applications fail, or increases in dose rates are needed in the applications (Tiryaki et al. 2010). In this sense, scientists around the world have turned to alternative control methods such as biological control to reduce the effects of pesticides used in agriculture and forestry or to limit their use. Bacterial insecticides used in the scope of microbial control have recently gained popularity due to their effectiveness, environmental safety, and easy production (Chattopadhyay et al. 2017). In this study, the culturable bacterial flora of *D. pini* was investigated to find possible new bacterial agents which can be used in biological control.

The highest mortality among all bacterial isolates was obtained from *P. mirabilis* O-2 with 100% mortality within ten days after application. This bacterium is in Enterobacteriaceae family and includes Gram-negative and facultative anaerobic rods. It is broadly found in soil and water. Also, it is known to be opportunistic pathogen in humans and isolated from urine, wounds, and other clinical specimens (Drzewiecka 2016). Up to now, this bacterium has been isolated from different insect species (Erdmann et al. 1986; Singh et al. 2015; Sancho et al. 1996). Especially, some species such as *P. mirabilis* in the genus of *Proteus* can be designated as potential pathogens since this group of bacteria has the possibility to grow in the hemocoel and

Table 2 The percent identity of the bacterial isolates with their the most related bacterial species or isolates using 16 S rRNA gene sequences based on Blast search in GenBank (Altschul et al. 1990; Benson et al. 2012)

Origin of isolate	Isolate name	Suggested species	GenBank ID Number	Query Coverage (%)	Identity (%)
Dead larvae	O-1	*Kluyvera intermedia* FE22	MH620738	100	99.02
		Kluyvera intermedia FM26	MH620736	100	98.95
		Kluyvera intermedia NCTC12125	LR134138	99	99.08
		Kluyvera intermedia FE15	MH620737	100	99.08
	O-2	*Proteus mirabilis* UFV 105	MN545923	100	100
		Proteus mirabilis HLJ1	KF811051	100	100
		Proteus mirabilis UFV 105	LR738920	100	100
		Proteus mirabilis UFV 131	MN546001	99	100
	O-3	*Klebsiella* sp. Y1(2011)	HQ616650	100	99.72
		Klebsiella oxytoca AR380	CP029128	99	99.79
		Klebsiella sp. SCT-5	KP262337	99	99.65
		Klebsiella oxytoca NCTC11356	LR133932	99	99.72
	O-4	*Bacillus* sp. (in: Bacteria) Z12	MG470665	100	100
		Bacillus halotolerans CCMM_B1214	MW303495	99	100
		Bacillus sp. PAMC26543	CP060193	99	100
		Bacillus halotolerans SY1836	MT271912	99	99.93
	O-5	*Pantoea agglomerans* 21-a blue	MN208199	100	99.79
		Pantoea agglomerans S19_PA1R	MK883100	99	99.72
		Enterobacter sp. snnu 6	MK087739	99	99.79
		Enterobacter ludwigii L23	JN700140	99	99.65
	O-6	*Serratia marcescens* OsEnb_HZB_G12	MN889394	100	99.79
		Serratia marcescens subsp. *sakuensis* XS 25 – 10	MT000048	100	99.79
		Serratia sp. CSBXZN6.10	LC484797	99	99.86
		Serratia marcescens BJV5	KM018333	99	99.86
	O-7	*Pseudomonas* sp. LJ20	KF515670	100	100
		Uncultured bacterium clone G9	MF631841	99	100
		Pseudomonas sp. NM	MG967453	99	100
		Pseudomonas taiwanensis P5	KM349421	99	100
	O-8	*Kluyvera intermedia* NCTC12125	LR134138	100	99.93
		Kluyvera intermedia JCM1238	NR112007	100	99.86
		Kluyvera intermedia FM26	MH620736	100	99.79
		Citrobacter sp. mixed culture J5-22	KR029237	100	99.79
	O-9	*Acinetobacter* sp. KU 011TH	MG372049	100	100
		Acinetobacter sp. KU 013TH	MG372050	100	100
		Acinetobacter lactucae QL-1	CP053391	100	99.93
		Acinetobacter dijkshoorniae LYC73-5	MH880847	100	99.93
	O-10	*Kluyvera intermedia* NCTC12125	LR134138	100	99.93
		Kluyvera intermedia JCM1238	NR112007	100	99.86
		Kluyvera intermedia FM26	MH620736	100	99.79
		Citrobacter sp. mixed culture J5-22	KR029237	100	99.79

cause generalized septicemia and the host death (Gouli et al. 2021). Tick pathogenic property of *P. mirabilis* has been reported and it has been suggested that it might hold promise for biological control of ticks (Brown et al. 1970; Samish and Rehacek 1999). Also, Kelly et al. (2013) showed that *P. mirabilis* caused entomopathogenic effects on African blue tick (*Rhipicephalus* (*Boophilus*) *decoloratus* (Koch, 1844) (Acarina: Ixodidae)) after application of 10^6 cfu/mL bacterial concentration. In addition, Maleki-Ravasan et al. (2014) suggested a symbiotic relationship between *P. mirabilis* and sandfly (*Phlebotomus papatasi* Scopoli (Diptera: Psychodidae)) and found that this bacterium was the most common species in larvae and pupae of sandfly which is the vector of *Leishmania major* associated with zoonotic cutaneous leishmaniasis. In this study, we observed 100% mortality on *D. pini* larvae within ten days after application of *P. mirabilis* O-2. This suggests that the isolate O-2 might have a good potential for the control of this pest.

The second highest mortality within all bacterial isolates was obtained from *K. intermedia* O-1 and O-8 with 80 and 93% mortality, respectively. The genus of *Kluyvera* is a group of bacteria involving Gram-negative, rod-shaped, and non-spore forming bacteria in the family of Enterobacteriaceae (Farmer et al. 1981). This genus consists of five species named as *K. intestini*, *K. intermedia*, *K. ascorbata*, *K. cryocrescens*, *K. intermedia* (Pavan et al. 2005; Tetz et

Table 2 (continued)

Origin of isolate	Isolate name	Suggested species	GenBank ID Number	Query Coverage (%)	Identity (%)
Healthy larvae	S-1	*Enterobacter* sp. IAUK3005	MK968092	100	99.86
		Pantoea agglomerans S19_PA1R	MK883100	100	99.86
		Enterobacter huaxiensis WTB145	MK734322	99	99.86
		Enterobacter ludwigii WTB95	MK241867	99	99.86
	S-2	*Bacillus pumilus* B7	KF641839	100	99.76
		Bacillus pumilus 161 (C2TL(A))	KF254674	100	99.76
		Bacillus pumilus L16	KX832718	99	99.86
		Bacillus sp. HY11(2010)	HM579802	99	99.86
	S-3	*Kluyvera intermedia* FM26	MH620736	100	99.79
		Kluyvera intermedia JCM1238	NR112007	99	99.86
		Kluyvera intermedia NCTC12125	LR134138	99	99.79
		Kluyvera intermedia 256	NR028802	99	99.79
	S-4	*Enterobacter cancerogenus* Gol8	MT263023	100	99.93
		Enterobacter cancerogenus gol6	MK426820	100	99.93
		Enterobacter cancerogenus C26	KJ410179	100	99.93
		Enterobacter asburiae MRY18-106	AP019533	100	99.86
	S-5	*Pantoea agglomerans* KM1	MT634720	100	97.16
		Pantoea vagans I-S-S2-8	MK398026	100	96.95
		Pantoea sp. PNS16	MK602409	100	96.95
		Pantoea sp. I-S-L2-10	MK398017	100	96.80
	S-6	*Pseudoclavibacter helvolus* JM89	MN758849	100	99.93
		Pseudoclavibacter terrae THG-MD12	NR145621	100	99.93
		Pseudoclavibacter sp. HP10L	KM187382	99	99.93
		Pseudoclavibacter sp. JSM 2,175,001	KM199858	100	99.86
	S-8	*Pseudoarthrobacter* sp. 206,447	MN726729	100	99.86
		Arthrobacter sp. B1.7	JN662533	100	99.86
		Arthrobacter sp. c138	AB167248	100	99.86
		Arthrobacter sp. c113	AB167242	100	99.86
	S-9	*Enterobacter* sp. DA 1	MG270576	100	99.79
		Enterobacter asburiae L1	CP007546	100	99.79
		Enterobacter cancerogenus JCM 3947	LC420103	100	99.65
		Enterobacter cancerogenus JCM 3943	LC420099	100	99.65

al. 2017). Some species of this genus can infrequently cause infections in humans and were isolated from various clinical specimens (Sarria et al. 2001). There are some studies showing the isolation of some species of *Kluyvera* genus from different insects (Ngoune et al. 2019; Ezemuoka et al. 2020; Mariño-Cárdenas et al. 2009). Muratoğlu et al. (2011) isolated and identified *K. cryocrescens* It3 from the European spruce bark beetle (*Ips typographus* s (L.) (Coleoptera: Curculionidae: Scolytinae)) but the isolate didn't show any insecticidal activity against *I. typographus*. Laurentis et al. (2014) investigated the biological effect of *K. ascorbata* in the development of the diamondback moth (*Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae)) showing the larval and pupal viability of the pest significantly reduced after feeding with cabbage leaves contaminated with this bacterium. Also, Crialesi et al. (2017) showed that *K. ascorbata* altered the metabolism of cabbage plants and this resulted in specific plant defense against *P. xylostella*. In this study, we for the first time showed that *K. intermedia* O-1 and O-8 might be insect pathogen considering its mortality values on *D. pini* larvae. This bacterium might be evaluated as a possible biocontrol agent against the common pine sawfly, but further studies are needed to test its pathogenicity and efficacy.

Many insect species such as aphids, scale insects and weevils harbor different species of the family Enterobacteriaceae which are maternally transmitted or found in a particular host species (Moran et al. 2005). In this study, the third highest mortality (86.6%) was obtained from *E. cancerogenus* S-4 isolated from healthy larvae. This bacterium which is formerly known as *E. taylorae* is a Gram-negative, facultative anaerobic, motile and consists of rod-shaped cells (bacil) in the family of Enterobacteriaceae. It is generally considered as opportunistic pathogen causing some infections in human (Garazzino et al. 2005; Wei et al. 2013; Davin-Regli et al. 2019). So far, a few bacteria in *Enterobacter* genus were isolated from various insects or invertebrates, some of which have agricultural importance (Blackburn et al. 2008; Sezen et al. 2007; Bahar and Demirbağ 2007; Zhang et al. 2021). The current study

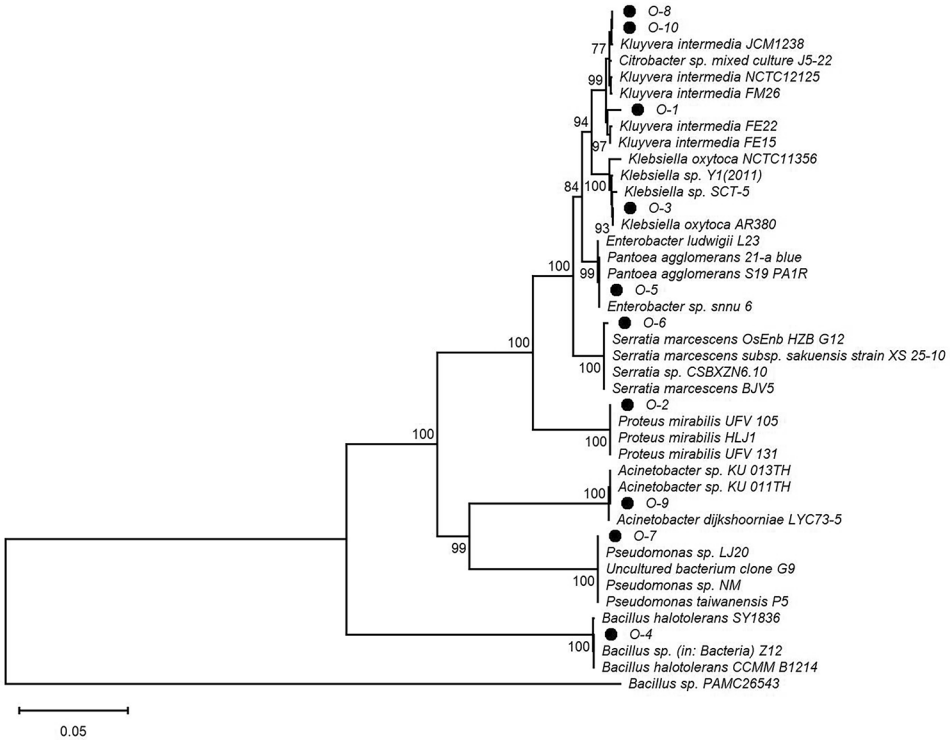

Fig. 1 A phylogram showing phylogenetic position of the bacterial isolates from dead larvae and their the most related bacterial species retrieved from GenBank based on Blast search. The tree was constructed using the neighbor-joining (N-J) analysis with p-distance correction by MEGA X software (Kumar et al. 2018). The bootstrap analysis was based on 1.000 pseudoreplicates and bootstrap values with > 70% were indicated. The bacterial isolates from dead *D. pini* larvae were indicated with the solid black circle. The scale on the bottom of the phylogram indicates the degree of dissimilarity

shows that some *Enterobacter* species, especially *E. cancerogenus* S-4, might be evaluated as a possible biocontrol agent against *D. pini* and further studies are needed to prove its pathogenicity or virulence and to elucidate their role in the common pine sawfly life.

Bacterial species in *Serratia* genus are Gram-negative, rod-shaped, and facultative anaerobes (Grimont and Grimont 2006). The type species of the genus is *S. marcescens* and it is related to infection of insects although it is not usually pathogenic to insects when present in the digestive tract in small numbers (Grimont and Grimont 2006; Sikorowski 1985; Pineda-Castellanos et al. 2015). Lauzon et al. (2003) found that the high dose application of *S. marcescens* to apple maggot flies caused 50% mortality after application of 24 h. Bahar and Demirbağ (2007) showed that *S. marcescens* Ol13 was found to cause 65% mortality on *Oberea linearis* L. (Coleoptera Cerambycidae) larvae under laboratory conditions. Bidari et al. (2017) found that *S. marcescens* significantly reduced the survival rates of *Polyphylla olivieri* (Laporte de Castelnau, 1840) (Coleoptera: Melolonthidae) larvae. In this study, we showed that *S. marcescens* O-6 caused 80% mortality on *D. pini* larvae and this isolate might be further investigated as a possible biocontrol agent of *D. pini*.

The genus of *Arthrobacter* includes a group of pleomorphic bacteria which can be found in many environments such as soil, aerial plant surfaces, and wastewater sediments and some members of the genus have effective roles in agriculture (Roy and Kumar 2020; Gobbetti and Rizzello 2014). Some species in the genus can utilize organic and inorganic compounds and these species can be used as a beneficial tool for bioremediation in agriculture. Some species have also been isolated from plant leaf surfaces and therefore predicted to have roles in plant growth-promoting activity (Roy and Kumar 2020; Scheublin et al. 2012). In the study of Gunner (1963), it was shown that *A. globiformis* has affect the biological oxidation of nitrogen. Although there are many studies about the isolation of some members of this genus from different insect species, there is no certain evidence that these bacteria or some certain species are insect pathogen (Eski et al. 2015; Barak et al. 2019; Sevim and Sevim 2021). In this study, we isolated *Arthrobacter* sp. S-8 from *D. pini* larvae and this bacterium showed 80% mortality against larvae of the pest. Considering of different roles of *Arthrobacter* species in nature and agriculture, it might be notable to further investigate the biological control potential of the isolate S-8, especially against *D. pini*.

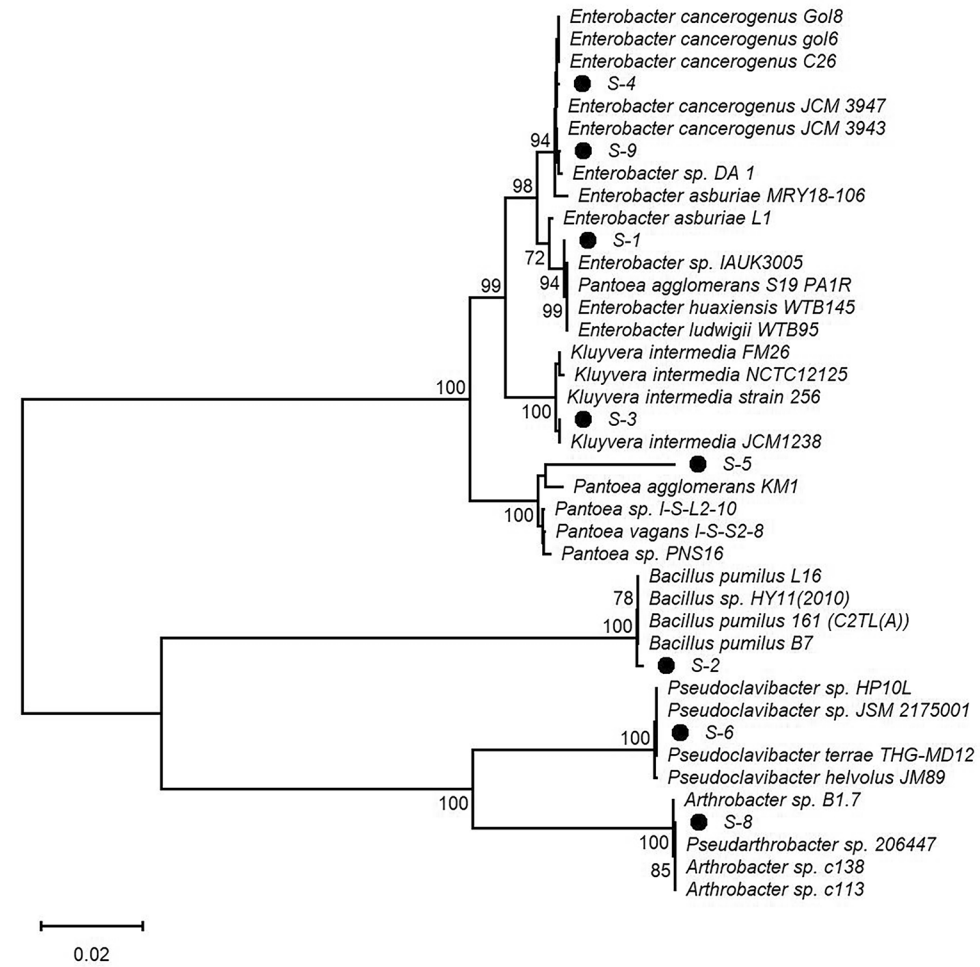

Fig. 2 A phylogram showing phylogenetic position of the bacterial isolates from healthy larvae and their the most related bacterial species retrieved from GenBank based on Blast search. The tree was constructed using the neighbor-joining (N-J) analysis with p-distance correction by MEGA X software (Kumar et al. 2018). The bootstrap analysis was based on 1.000 pseudoreplicates and bootstrap values with > 70% were indicated. The bacterial isolates from healthy *D. pini* larvae were indicated with the solid black circle. The scale on the bottom of the phylogram indicates the degree of dissimilarity

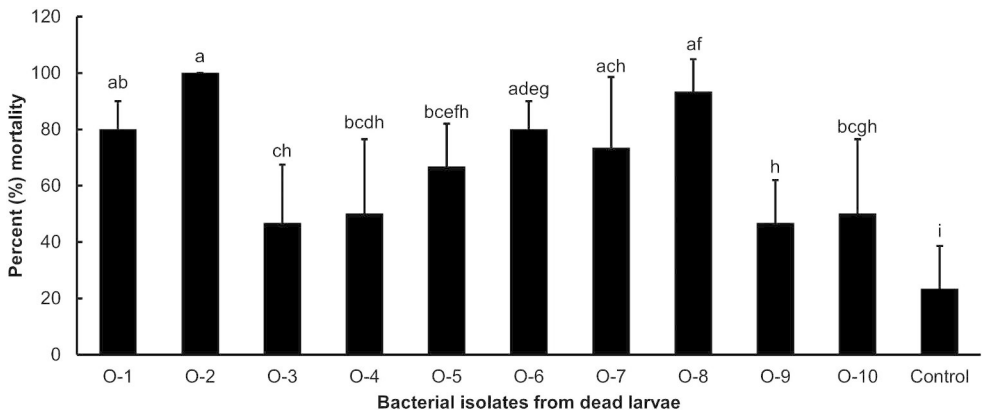

Fig. 3 Percent (%) mortalities of the bacterial isolates from dead larvae within ten days after inoculation. The bacterial concentration of 1.8×10^9 cfu/mL for each isolate were applied to *D. pini* larvae. Mortality data were calculated as percent data based on the Abbott's formula (Abbott 1925). Bars show standard deviation. Different letters over mortality columns represent statistically significant difference among the bacterial isolates with respect to mortality. O-1, O-8, and O-10; *Kluyvera intermedia*, O-2; *Proteus mirabilis*, O-3; *Klebsiella oxytoca*, O-4; *Bacillus* sp., O-5; *Pantoea agglomerans*, O-6; *Serratia marcescens*, O-7; *Pseudomonas* sp. and O-9; *Acinetobacter* sp

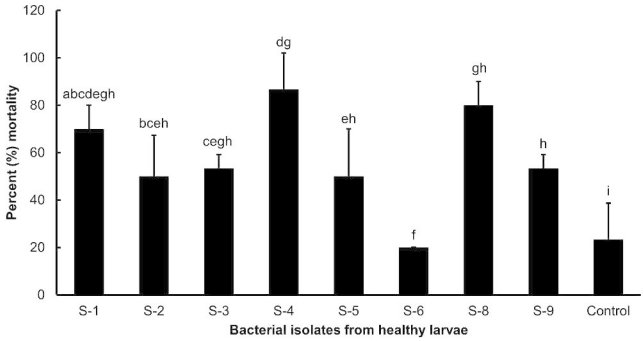

Fig. 4 Percent (%) mortalities of the bacterial isolates from healthy larvae within ten days after inoculation. The bacterial concentration of 1.8×10^9 cfu/mL for each isolate were applied to *D. pini* larvae. Mortality data were calculated as percent data based on the Abbott's formula (Abbott 1925). Bars show standard deviation. Different letters over mortality columns represent statistically significant differences among the bacterial isolates with respect to mortality. S-1; *Enterobacter* sp., S-2; *Bacillus pumilus*, S-3; *Kluyvera intermedia*, S-4; *Enterobacter cancerogenus*, S-5; *Pantoea agglomerans*, S-6; *Pseudoclavibacter* sp., S-8; *Arthrobacter* sp. and S-9; *Enterobacter* sp

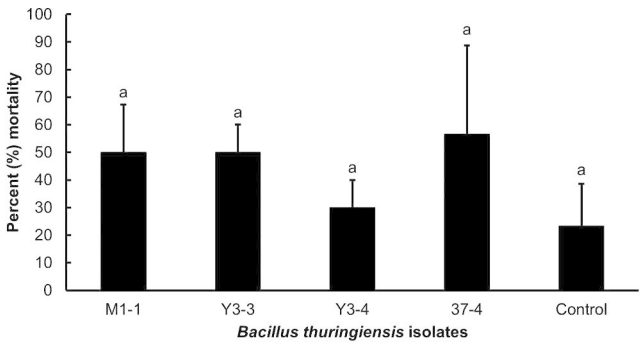

Fig. 5 Percent (%) mortalities of *B. thuringiensis* isolates within ten days after inoculation. The bacterial concentration of 1.8×10^9 cfu/mL for each isolate were applied to *D. pini* larvae. Mortality data were calculated as percent data based on the Abbott's formula (Abbott 1925). Bars show standard deviation. Different letters over mortality columns represent statistically significant differences among the bacterial isolates with respect to mortality

We also tested four different *B. thuringiensis* isolates against larvae of *D. pini*. Although Bt isolates showed moderate pathogenicity, all of them were not statistically different from the control group. Different *Bt* isolates produce different insecticidal protein toxins which have almost the same structure. Up to now, more than 850 Cry proteins have been described from 78 families (Cry1 to Cry78) (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). Due to their different toxin contents, different *Bt* strains differ in host range. For example, Cry1 and Cry2 are highly toxic to Lepidopteran insects (Palma et al. 2014; Zheng et al. 2017). The reason why *Bt* strains did not show effective pathogenicity on *D. pini* larvae in our study may be since these isolates did not contain target toxins to *D. pini*. However, this needs to be proven by testing these isolates on other pests from different orders.

Bacterial endosymbionts associated with insects are usually passed from mother to offspring via vertical transmission and have many functions in insects. For example, they are known to be responsible for the digestion of nutrients in the gut, the synthesis of essential amino acids or vitamins, defense against pathogens or parasites, environmental adaptation, and population dynamics of insects (Su et al. 2013). In addition, it is suggested that the number of insect pests in agriculture and forestry can be reduced by manipulating the microbiota (Paniagua Voirol et al. 2018). For instance, Harada and Ishikawa (1997) isolated different species of bacteria from the gut of aphids (*Acyrthosiphon pisum* (Harris) (Hemiptera Aphididae)) and cultured them. Then, they mixed each gut bacteria with nutrients and returned it to the host. Finally, they observed that these bacteria tended to multiply rapidly in the gut and eventually killed the host. In this study, various gut bacteria were isolated from healthy *D. pini* larvae, and some of these (especially *E. cancerogenus* S-4 and *Arthrobacter* sp. S-8) caused mortality when they were given to the host with nutrients. Hereby, it can be important to investigate the possibilities of the use of bacterial species isolated from the gut of healthy *D. pini* larvae in the biological control of *D. pini*.

In conclusion, we isolated and identified possible insect pathogenic bacteria from *D. pini* larvae and evaluated their biological control potential in artificial infection experiment. The obtained result might be beneficial for the future biocontrol programs of the pest. More detailed biochemical and molecular studies are needed to identify some of isolates (O-4, O-7, O-9, S-1, S-6, S-8, and S-9) at species level. The field studies and dose-response tests are needed to determine the field efficacy of the most effective isolate (*P. mirabilis* O-2). The potential risks associated with the use of a novel biocontrol agent for pest control should be considered and studies related to safety assessment should be performed. In addition, further studies are needed to elucidate the roles of non-pathogenic strains in *D. pini* life cycle.

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Authors' contributions Tayyib çelik participated in material collection, bacterial isolation, identification, infection experiments and revising the manuscript. Ali Sevim participated in almost all parts of the study such as the study conception, design, material preparation, data collection, analysis and writing the manuscript. All authors read and approved the final version manuscript.

Declarations

Conflicts of interest/Competing interests The authors have no con-

flicts of interest to declare that are relevant to the content of this article.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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