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## Isolation, identification and characterization of biotechnologically important bacteria from microflora of *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae)

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### ABSTRACT

The chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) is one of the most important insect pests of chestnut. The aim of this study was to isolate and characterize bacteria from *D. kuriphilus* to obtain new microbial agents for both biological control and other biotechnological applications. *D. kuriphilus* larvae were collected from chestnut fields located in Bursa and Yalova provinces of Marmara Region of Turkey during May–July 2014. Four bacterial isolates were obtained from *D. kuriphilus*. According to their morphological, biochemical and molecular properties, these isolates were identified as *Staphylococcus saprophyticus* (Dk1), *Paenibacillus* sp. (Dk2), *Pseudomonas fluorescens* (Dk3) and *Paenibacillus* sp. (Dk4). To the best of our knowledge, this is the first study on the bacterial flora of *D. kuriphilus*. In our study, the potential of these isolates as a biological control agent against different hazardous pests and other possible biotechnological applications of importance were discussed under the light of literature.

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## Introduction

Insects are considered the most successful group of animals, in terms of both diversity and survivability in various ecological niches [1]. The microflora of an insect's body contains many important bacterial species of biotechnological importance. These bacteria utilize various organic polymers and may belong to the groups of methanogenic and nitrogen-fixing bacteria [2,3]. The gut microflora of insects has also been reported to contribute to important processes, such as the synthesis of pheromones and vitamins, degradation of pesticides and protection against pathogen attack [4,5]. That is why both the total microflora and the gut microflora of insects are a good source of novel isolates of potential biotechnological importance. Furthermore, the insect gut is estimated to contain 10-fold more microbes than the total cells of the insects and 100-fold more microbial genes than animal genes [1,6].

The chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), is one of the most important pests of chestnuts worldwide. It is native to China and has been detected in various countries from Asia to America and Europe. Reported for the first time

in Turkey in 2014, it is currently present in Yalova and Bursa Regions [7]. Considered as a major pest of *Castanea* species worldwide, *D. kuriphilus* causes galls and can reduce the fruit production.

Up to now, chemical substances such as methamidophos, omethoate and dichlorvos have been used to control this pest [8]. However, chemical control has hazardous effects on the environment. Although there are a lot of biological control studies on the use of *Torymus sinensis* Kamijo (Hymenoptera, Torymidae) as a classical biological control agent against *D. kuriphilus*, to date, the isolation and characterization of bacteria from *D. kuriphilus* has remained a neglected area of research.

Recently, there has been an increasing interest in the discovery of more effective and pathogenic bacterial control agents against harmful insects. The aim of this study was to isolate and characterize bacteria from *D. kuriphilus* to obtain new microbial agents to be used for biological control. Other potential biotechnological applications of the bacterial isolates have also been discussed. To the best of our knowledge, this is the first study to investigate the bacterial flora of *D. kuriphilus*.

## Materials and methods

### Collection of insects

*D. kuriphilus* larvae were collected from chestnut fields located in Bursa and Yalova provinces of Marmara Region of Turkey during May–July 2014. The larvae were collected in aseptic conditions and immediately transported to the laboratory.

### Isolation of bacteria

Living and dead larvae were distinguished by macroscopic examination. The larvae were surface-sterilized in 70% alcohol and then washed three times in sterile distilled water and homogenized in nutrient broth media (Merck, Darmstadt, Germany) by using a glass tissue grinder. Suspensions were diluted (from  $10^{-1}$  to  $10^{-8}$ ) and 0.1 mL of each suspension was plated on nutrient agar. Plates were incubated at 30 °C for 2–3 days. After the incubation period, the plates were examined and bacterial colonies were selected. The colonies were then purified by subculture on plates.

### Identification of bacterial isolates

Bacterial isolates were examined and identified based on their morphological (cell morphology, endospore formation and mobility) and biochemical properties (Gram staining, oxidase and catalase tests). In addition, the biochemical properties of the bacterial isolates were also determined using the Analytical Profile Index API 20E, API 50CH and API STAPH test systems (BioMerieux, Marcy l'Etoile, France).

### Polymerase chain reaction (PCR)

Bacterial colonies were inoculated into nutrient broth and incubated for approximately 18 h at 30 °C prior to DNA extraction. At the end of the incubation time, the bacterial cells were collected from the culture medium by centrifugation. Then, genomic DNA was isolated using a Genomic DNA Purification Kit (Promega, Madison, WI, USA) in accordance with the manufacturer's recommendations. The oligonucleotide primers (Macrogen, Amsterdam, The Netherlands) of 27F (5'-AGAGTTT-GATCMTGGCTCAG-3' as forward) and 1492R (5'-GGY-TACCTTGTTACGACTT-3' as reverse) were used for amplification of the 16S rRNA genes. The PCR mixture contained 5 µL of 5× *Taq* DNA polymerase reaction buffer (Thermo Fisher SCIENTIFIC, Paisley, UK), 200 µmol/L of each deoxyribonucleoside triphosphate (dNTP), 10 pmol of primers, 1.5 U of *Taq* DNA polymerase (Thermo Fisher SCIENTIFIC, Paisley, UK), 3 mmol/L of

MgCl<sub>2</sub> and 5 µL of genomic DNA in a final reaction volume of 50 µL. The PCR was performed under the following conditions (T100™ Thermal Cycler, Bio-Rad, Hercules, CA, USA): 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) and a final extension at 72 °C for 10 min. Finally, the PCR products were analyzed by electrophoresis in 1% agarose gel and then visualized under ultraviolet (UV) light by staining with ethidium bromide. The PCR products were sequenced by RefGen Biotechnology Laboratory (Ankara, Turkey). The same primer pairs were used for sequencing. The obtained sequences were used for identification of the isolates and phylogenetic analyses. BLAST (Basic Local Alignment Search Tool) searches were performed using the NCBI (National Center for Biotechnology Information) GenBank database to confirm the isolate identification [9].

The evolutionary relationships of the four bacterial isolates were evaluated. Cluster analyses of the sequences were performed using BioEdit (version 7.09) with Clustal W followed by neighbour-joining analysis on aligned sequences performed with MEGA 6.0 software [10]. The reliability of the dendrograms was tested by bootstrap analysis with 1000 replicates using MEGA 6.0.

## Results and discussion

Recently, there has been an increasing interest in finding more effective and pathogenic bacterial control agents against harmful insects. Although there are a lot of biological control studies on *D. kuriphilus* to date, the isolation and characterization of bacteria from *D. kuriphilus* have been neglected. In the present study, four bacterial isolates, two spore-forming ones (isolates Dk2 and Dk4) and two non-spore-forming ones (isolates Dk1 and Dk3), were isolated from *D. kuriphilus*. Some morphological and biochemical characteristics of these bacterial isolates are summarized in Table 1. In addition to conventional tests, API test results of bacterial isolates are given in Tables 2–4.

The partial 16S rRNA gene sequences generated in this study were deposited in the GenBank database

**Table 1.** Some morphological and biochemical characteristics of *D. kuriphilus* isolates.

Tests	Dk1	Dk2	Dk3	Dk4
Colony colour	Cream	Cream	Cream	Cream
Cell shape	Cocci	Rod	Rod	Rod
Gram stain	+	+	–	+
Spore stain	–	+	–	+
Motility	–	+	–	+
Catalase test	+	+	+	+
Oxidase test	–	–	+	–

+, positive; –, negative.

**Table 2.** API 50CH test results for *D. kuriphilus* isolates.

Tests	Dk2	Dk4
Glycerol	+	+
Erythritol	—	—
D-arabinose	—	—
L-arabinose	+	+
D-ribose	+	+
D-xylose	+	+
L-xylose	—	—
D-adonitol	—	—
Methyl- $\beta$ -D-xylopyranoside	+	—
D-galactose	+	—
D-glucose	+	+
D-fructose	+	+
D-mannose	+	+
L-sorbose	—	—
L-rhamnose	—	—
Dulcitol	—	—
Inositol	—	—
D-mannitol	+	+
D-sorbitol	—	—
Methyl- $\alpha$ -D-mannopyranoside	—	—
Methyl- $\alpha$ -D-glucopyranoside	wp	—
N-acetylglucosamine	+	—
Amygdalin	+	+
Arbutin	+	+
Esculin (ferric citrate)	+	+
Salicin	+	+
D-cellobiose	+	+
D-maltose	+	+
D-lactose (bovine origin)	+	+
D-melibiose	+	+
D-saccharose	+	+
D-trehalose	+	+
Inulin	+	wp
D-melezitose	—	—
D-raffinose	+	+
Starch	+	+
Glycogen	+	+
Xylitol	—	—
Gentiobiose	wp	+
D-turanose	—	—
D-lyxose	—	—
D-tagatose	—	—
D-fucose	—	—
L-fucose	—	—
D-arabitol	—	—
L-arabitol	—	—
Potassium gluconate	—	—
Potassium 2-ketogluconate	—	—
Potassium 5-ketogluconate	—	—

+, positive; —, negative; wp, weak positive.

under the following accession numbers: Dk1, KT377432; Dk2, KT377433; Dk3, KT377434 and Dk4, KT377435 (Table 5). A phylogenetic tree was constructed using the neighbour-joining method (Figure 1). The isolates showed a similarity of 97%–99% compared with other species.

### *Staphylococcus saprophyticus*

The Dk1 isolate is Gram-positive, non-spore-forming, non-motile, cocci-shaped, catalase-positive and oxidase-negative, with cream-coloured colonies on nutrient agar. Based on the conventional tests, the API 20E, API 50CH, API STAPH, the morphological and biochemical characteristic and the partial 16S rRNA sequence analysis, this

**Table 3.** API 20E test results for *D. kuriphilus* isolates.

Tests	Dk1	Dk3
$\beta$ -galactosidase	+	—
Arginine dihydrolase	+	—
Lysine decarboxylase	—	+
Ornithine decarboxylase	—	—
Trisodium citrate	+	—
H <sub>2</sub> S (sodium thiosulfate)	—	—
Urease	+	wp
L-tryptophan	—	wp
Indole	—	—
VP (sodium pyruvate) test	+	+
Gelatinase	+	+
Glucose fermentation	+	—
D-mannitol fermentation	+	—
Inositol fermentation	—	—
D-sorbitol fermentation	—	—
L-rhamnose fermentation	—	—
D-saccharose fermentation	—	—
D-melibiose fermentation	—	—
Amygdaline fermentation	—	—
L-arabinose fermentation	—	—

+, positive; —, negative; wp, weak positive.

**Table 4.** API STAPH test results for the Dk1 isolate.

Tests	
Glucose	+
Fructose	+
Mannose	+
Maltose	+
Lactose	+
Trehalose	+
Mannitol	+
Xylitol	—
Melibiose	—
Nitrate to nitrite	+
Alkaline phosphatase production	+
Acetyl methyl carbinol production	—
Raffinose	—
Xylose	—
Saccharose	+
Acid from alpha-methylglucoside	—
Acid from N-acetylglucosamine	+
Arginine dihydrolase	+
Urease	+

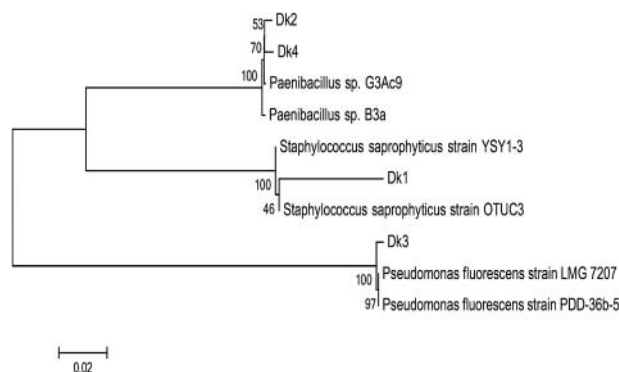
+, positive; —, negative.

**Table 5.** GenBank accession numbers and identification of bacterial isolates according to partial 16S rRNA gene sequences.

Isolates	GenBank	Conclusion	16S rRNA sequence similarity (%)	Accession numbers
Dk1	KT377432	<i>Staphylococcus saprophyticus</i>	97	GU197531
Dk2	KT377433	<i>Paenibacillus</i> sp.	99	KF465974
Dk3	KT377434	<i>Pseudomonas fluorescens</i>	99	GU198115
Dk4	KT377435	<i>Paenibacillus</i> sp.	99	EU558285

isolate was identified as *Staphylococcus saprophyticus*. Isolate Dk1 has been previously isolated from red imported fire ant larvae, *Solenopsis invicta*, and mealy bug, *Rhizoecus amorophophalli* [11,12].

One of the few reports on the potential of *S. saprophyticus* as a biological control agent demonstrates



**Figure 1.** Neighbour-joining tree of bacterial isolates from *D. kuriphilus*. The dendrogram was constructed by MEGA 6.0 software based on the partial sequences of the 16S rRNA gene. Bootstrap values shown next to nodes are based on 1000 replicates. The scale on the bottom of the dendrogram shows the degree of dissimilarity.

that *S. saprophyticus* can be used for the control of cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) [13]. As pointed out by Miranda-Miranda et al. [13,14], under certain conditions, *Staphylococci* can cause a lethal infection in fully engorged ticks, but the underlying mechanism by which these bacteria infect the engorged females have yet to be clarified to better assess the potential of *S. saprophyticus* as an alternative method for control of the cattle tick and tick-transmitted diseases.

Apart from biological control, *S. saprophyticus* has been used in some other biotechnological applications as well. For example, İlhan et al. [15] report that *S. saprophyticus* can be used in the removal or recovery of heavy metal ions, especially lead and chromium ions, from industrial waste waters by biosorption. In another study, lysostaphin, a staphylococcal bacteriolysin with potential clinical applications, was produced and was shown to be potentially useful as a biopreservative in the food industry as well [16].

*S. saprophyticus* is a good producer of slime (biofilm material). It is well known that a wastewater treatment system based on a trickling filter consists of a fixed bed of rocks containing slime material. That is why slime-producing bacteria might be useful in this type of wastewater treatment systems [17,18].

In addition to the above-mentioned biotechnological potential, some strains of *S. saprophyticus* (for example *S. saprophyticus* M36) have high activity of organic solvent-stable lipase [19]. Organic solvent-stable lipase is used in various industries such as food, dairy, pharmaceutical, cosmetic, detergents, textile and biodiesel industry as well as in the synthesis of fine chemicals and new polymeric materials and in environmentally friendly industries [20,21].

### *Paenibacillus* sp.

The Dk2 and Dk4 isolates are motile, Gram-positive, rod-shaped, catalase-positive, oxidase-negative, spore-forming, with cream-coloured colonies on nutrient agar. Based on the morphological and biochemical tests, the API system and 16S rRNA gene sequence analysis, these two isolates were identified as *Paenibacillus* sp. Some members of the genus *Paenibacillus* have been associated with insect mortality in one way or another [22]. There are *Paenibacillus* species (e.g. *P. popilliae*, *P. lentimorbus* and *P. larvae*) that are pathogenic for insects. Isolates Dk2 and Dk4 have also been isolated from different insects in previous studies and have been investigated for pathogenic potential in insects [23,24]. In previous studies, *Paenibacillus* species have been used as biological control agents. For example, *P. alvei* strain K165 has been applied against the cotton black root rot pathogen, *Thielaviopsis basicola* [25]; *P. alvei* strain K165, against Verticillium wilt of olive trees [26]; *P. polymyxa* E681, in sesame seed pelleting for prevention of damping-off diseases and wilt [27] and *P. polymyxa* cw, against tomato and red pepper powdery mildew [28]. On the other hand, *Paenibacillus* species are some of the most industrially significant facultative anaerobic bacteria. Their natural habitat includes the soil, the rhizosphere and the roots of crop plants, and marine sediments [29]. Although we have limited knowledge about their genomic information, the academic interest towards their ecological and biotechnological roles has increased in the last 20 years or so. For example, *P. polymyxa* has a lot of different features like nitrogen fixation, promotion of plant growth, solubilization of phosphorus, production of exopolysaccharides, hydrolytic enzymes, antibiotics, cytokinins and other valuable and useful compounds, such as optically active 2,3-butanediol. Other *Paenibacillus* strains boost the flocculation and enhance the soil porosity [29].

### *Pseudomonas fluorescens*

The Dk3 isolate is Gram-positive, non-spore-forming, non-motile, rod-shaped, catalase- and oxidase-positive, with cream-coloured colonies on nutrient agar. Based on the applied battery of tests, it was identified as *Pseudomonas fluorescens*. *P. fluorescens* strains have been reported to produce metabolites with insecticidal properties, such as HCN and the lipopeptides viscosin and orfamide [30,31]. Interestingly, some of the plant root-colonizing pseudomonads are pathogenic to insects and have been proposed for potential exploitation as novel bioinsecticides against root-feeding insects (for review, see [32]). Kupferschmid et al. [32] discuss the



accumulated knowledge about the occurrence and the molecular basis of insecticidal activity in pseudomonads and how this trait could be used in novel root-based approaches for insect control in an integrated framework of pest management. *P. fluorescens* has been isolated from different insects in previous studies and has been investigated for pathogenicity in insects [33,34]. Although there is no literature on the use of *P. fluorescens* as a biological control agent against *D. kuriphilus*, it has been used as a biocontrol agent against some other hazardous organisms, especially phytopathogenic fungi and bacteria. Daval et al. [35] report that *P. fluorescens* Pf29 can be used for control of the fungus *Gaeumannomyces graminis* var. *tritici* (Ggt) on wheat roots. On the other hand, some strains of *P. fluorescens* have also been used as biocontrol agents against *Rhizoctonia solani* in tobacco (*Nicotiana tabacum*) seed beds [36], common scab symptoms in the field [37] and three phytopathogenic fungi, *Phythium ultimum*, *Macrophomina phaseolina* and *Piricularia oryzae* [38].

Apart from biocontrol studies, *P. fluorescens* has been used in many other biotechnological applications such as biosynthesis of massetolide, a cyclic lipopeptide antibiotic [39], detoxification and bioremediation of metals such as Al, Ga, and Ca [40,41], fluorescent pigment production [42] and phenazine biosynthesis [43]. All these examples of biological pest control and other biotechnological applications of the isolated bacterial strains and their closely related species strongly suggest that the four bacterial isolates from *D. kuriphilus* studied here might also possess such valuable properties.

## Conclusions

To the best of our knowledge, there has been no report on the isolation and characterization of bacteria from *D. kuriphilus* until now. The four isolates identified as *S. saprophyticus* (Dk1), *Paenibacillus* sp. (Dk2 and Dk4) and *Pseudomonas fluorescens* (Dk3) belong to species and genera of known practical importance. The survey of relevant literature suggested that all four isolates have a promising potential for use in different biotechnological applications. Future studies should focus on investigation of their biological control properties and possibilities for biotechnological applications.

## Disclosure statement

The authors declare that they have no competing interests.

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