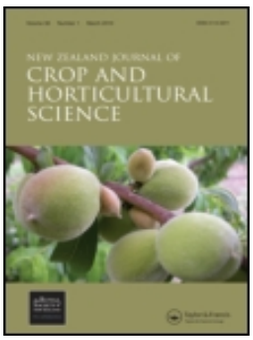

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# Identification and pathogenicity of entomopathogenic bacteria from common cockchafer, *Melolontha melolontha* (Coleoptera: Scarabaeidae)

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**Abstract** *Melolontha melolontha* larvae are susceptible to several pathogens indigenous to the area in which these insects occur in Turkey. We isolated and identified seven bacterial strains from *M. melolontha* and evaluated their pathogenic activity during three hazelnut seasons from 2002 to 2004 on larvae of *M. melolontha*. Using various morphological, physiological, and biochemical characteristics in detail, bacterial isolates were identified as *Pseudomonas* sp., *Bacillus thuringiensis*, *Pseudomonas* sp., *Enterobacter* sp., *B. sphaericus*, *Acinetobacter* sp., and *B. weihenstephanensis*. The insecticidal activity of isolates at $1.8 \times 10^9$ bacteria/ml dose, within 10 days on the larvae of *M. melolontha* are 40% for *Pseudomonas* sp., 80% for *Bacillus thuringiensis*, 50% for *Pseudomonas* sp., 20% for *Enterobacter* sp., 60% for *B. sphaericus*, and 80% for *B. weihenstephanensis*. We also purified crystals from *B. thuringiensis* and *B. sphaericus* and tested the insecticidal activity on the larvae of *M. melolontha*. In crystal protein bioassays, the highest insecticidal effect detected was 70% with crystals of *B. thuringiensis*. Our results indicate that indigenous *B. thuringiensis* and *B. weihenstephanensis* isolates and crystal of *B. thuringiensis* may be valuable as biological control agents.

**Keywords** *Melolontha melolontha*; bacterial flora; biological control; insecticidal activity

## INTRODUCTION

A complex of pests cause damage on hazelnuts (*Corylus* sp.) in North America, Europe, and Turkey (Aliniazee 1999). For production and exportation, Turkey is first among all hazelnut producing countries. However, Turkey lags far behind many other countries in its yield per unit area. This is largely attributable to the many agricultural pests of hazelnut which can not be effectively controlled in Turkey. The common cockchafer, *Melolontha melolontha* L. is one of the most important insect pests of hazelnuts in the Black Sea Region of Turkey, causing 15–20% damage annually (Froschle 1994; Luisa & Mauro 1996; Vlug 1996; Sezen 2004).

At present two pesticides, endosulfan and chlorpyrifos-ethyl, are used in Turkey to control *M. melolontha*. Increasing interest in developing environmentally safe pest control methods has inspired us to study the potential of bacteria for controlling *M. melolontha*. There are no previous studies of bacterial flora as biological control agents of the common cockchafer, and this insect is very attractive for biological control studies. Infected insect larvae present certain symptoms that suggest bacterial infection; they become flaccid, lethargic, and stop eating (Osborn et al. 2002). Various bacterial insect pathogens are used successfully in microbial control of insects (Thiery & Frachon 1997; Sezen et al. 2001, 2004). Here, we report on the isolation and identification of bacteria found in living and dead *M. melolontha*. We tested the insecticidal activities of these bacterial isolates and crystals and crystal-spore mixtures of *B. thuringiensis* and *B. sphaericus* strains against the larvae of *M. melolontha* as possible biological control agents.

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## MATERIALS AND METHODS

### Collection of samples

Larvae and adults of the common cockchafer were collected from the vicinity of Trabzon, in Turkey during the period 2002–04. Adults were collected weekly from the beginning of May and larvae were collected daily from the beginning of March. Collected insects were brought to the Microbiology Laboratory at Biology Department, Karadeniz Technical University, Trabzon/Turkey.

### Quantitative analysis of bacteria from larvae and adults

First, live and dead larvae and adults were surface sterilised with 70% alcohol and homogenised in a homogeniser containing 5 ml of sterilised phosphate buffer solution (PBS, pH 7.4). The suspensions which were obtained from larvae and adults bodies were filtered twice through two layers of cheesecloth to remove debris (Poinar 1978). The suspension was diluted to $10^{-8}$ (Christine & Ted 1992). One hundred $\mu$l of each suspension was plated on nutrient agar. Plates were incubated at 30°C for 24 h. After incubation, the total number of bacteria in larvae and adults was determined by counting the number of colonies on the plates which were inoculated with diluted bacterial suspensions.

### Isolation and identification of bacteria from *M. melolontha*

Living and dead larvae and adults of field-collected *M. melolontha* were surface sterilised with 70% alcohol. In some instances, attempts were made to isolate bacteria from hemolymph; in other instances the insect body was triturated. Bacterial isolates were prepared in one of two ways. The larval suspension was heated at 80°C for 10 min in a water bath to eliminate the nonspore-forming bacteria (Ohba & Aizawa 1986; Lee et al. 1992; Thiery & Frachon 1997). One hundred $\mu$l of the heat-treated samples were spread on nutrient agar plates and incubated at 28°C for 48–96 h (Lee et al. 1995), then examined, and bacterial colonies were selected based on colour and morphology. Alternately, the suspension was applied directly to nutrient agar plates. The isolation procedures were carried out during three hazelnut seasons from 2002 to 2004. Plates were incubated at 28°C for 48–96 h. Isolates were separated based on colour and morphology of the colonies. Pure cultures of bacterial colonies were then prepared, and these cultures were identified by various tests. Tests such as use of organic compounds, spore formation, NaCl tolerance, optimum pH, optimum temperature, catalase test, oxidase test, and gelatine hydrolysis were performed for all isolates.

The identification procedure of isolated bacteria was done according to "Bergey's Manual of Systematic Bacteriology 1 and 2" (Krieg & Holt 1986; Sneath et al. 1986).

### Experimental infections for bacterial isolates

Non-spore forming bacteria and spore-forming bacteria were incubated in nutrient broth medium at 30°C for 18 h and at 30°C for 72 h (for sporulation) respectively. After incubation, the bacterium density was measured as 1.89 at optical density$_{600}$ ($1.8 \times 10^9$ cfu) (Ben-Dov et al. 1995; Moar et al. 1995). Five ml of this culture was centrifuged at 3000 rpm for 10 min. The pellet was resuspended in 5 ml of sterilised phosphate buffer solution and used in bioassays.

### Isolation and experimental infections for crystal proteins of *Bacillus* sp.

After incubation, *B. thuringiensis* and *B. sphaericus* were collected and lysed. The mixture of spores and crystals was suspended in 0.5*M* NaCl and centrifuged at 13 800*g* for 5 min. The pellet was resuspended in 1% SDS and 0.001% β-mercaptoethanol, boiled for 10 min, and then centrifuged at 13 800*g* for 10 min (Sivripoulou et al. 2000). Supernatant, containing mainly crystal proteins, was removed, measured by spectrophotometry (OD$_{595}$), and used (100 $\mu$g/ml) for infectivity tests.

### General conditions for experimental infections

A diet was prepared from roots of *Corylus* sp. and collected field soil. The diet was placed into individual sterilised glass containers (80 mm in diam.). Bacterial isolates and crystal proteins prepared in PBS were applied to the surface of the diet. Ten each of second and third instar larvae were placed on the diet in containers. Containers were kept at 26 ± 2°C and 60% room humidity on a 12:12 h photo regime, with the diet changed after eating (Lipa et al. 1994). The mortalities of larvae were recorded every 24 h and all dead larvae were removed from containers. Negative control which has sterile water was also included in infectivity tests. At least 30 larvae were assayed for each isolate. All bioassays were repeated 3 times on different occasions. Mean mortality by isolate were analysed using one-way analysis of variance (ANOVA) and compared by least significant difference (LSD) test (Minitab 1997).

## RESULTS AND DISCUSSION

There has recently been an increasing interest in finding an effective and safer biological control agent against hazardous insects. Although there are many biological control studies on *M. melolontha*, there has been no study on the isolation and characterisation of bacterial flora of *M. melolontha* as potential biological control agents to date.

We found only seven different isolates based on colony morphology among all the bacterial suspensions from the *M. melolontha* insects. These isolates were characterised for morphology, spore formation, nutritional features, and physiological and biochemical characteristics (Table 1). All of the isolates were rod-shaped, occurring singly, commonly 0.95–8.55 $\mu$m in length. Three isolates (Mm2, Mm5, and Mm7) were gram positive and the remainder were gram negative. Motility was observed in all isolates. Whereas the catalase test was positive for all isolates, the oxidase test was negative for isolates Mm1, Mm2, Mm4, Mm6,

**Table 1** Morphological, physiological, and biochemical characteristics of the bacterial isolates of *Melolontha melolontha*. (ND, not determined.)

| Tests | Isolate | | | | | | |
|---|---|---|---|---|---|---|---|
| | Mm1 | Mm2 | Mm3 | Mm4 | Mm5 | Mm6 | Mm7 |
| Colony colour | cream | cream | cream | cream | cream | cream | cream |
| Shape of bacteria | rod | rod | rod | rod | rod | rod | rod |
| Gram stain | – | + | – | – | + | – | + |
| Spore stain | – | + | – | – | + | – | + |
| Shape of spore | ND | elipsoid | ND | ND | elipsoid | ND | elipsoid |
| Location of spore | ND | central | ND | ND | central | ND | terminal |
| Length ($\mu$m) | 2.09–3.8 | 3.13–4.75 | 0.95–1.42 | 0.95–1.9 | 2.85–8.55 | 1.04–1.42 | 2.85–7.6 |
| Wideness ($\mu$m) | 0.8–0.95 | 0.85–1.04 | 0.47–0.76 | 0.66–0.85 | 0.95–1.04 | 0.76–0.85 | 0.85–0.95 |
| Motility | + | + | + | + | + | + | + |
| Nitrate reduction | - | + | + | + | + | – | + |
| Hydrolysis of starch | - | + | - | - | - | – | + |
| Oxidase | - | - | + | - | + | – | – |
| Hydrolysis of gelatine | + | + | + | - | + | – | + |
| Hydrolysis of urea | - | - | - | - | - | + | + |
| Citrate use | + | + | + | + | - | + | + |
| Propionate use | - | - | + | - | - | + | – |
| Indole test | - | - | - | + | - | - | – |
| Methyl red test | - | + | + | + | + | + | + |
| Voges proskauer test | + | - | + | - | - | - | + |
| Growth in 7% NaCl | + | - | + | + | - | + | – |
| Growth in 12% NaCl | - | ND | - | - | - | W+ | ND |
| Growth with lysozyme | + | + | + | + | + | + | + |
| Growth at 4°C | ND | ND | + | ND | ND | ND | ND |
| Growth at 40°C | ND | + | ND | ND | ND | ND | + |
| Growth at 41°C | ND | ND | + | ND | ND | ND | ND |
| Growth at 50°C | ND | - | ND | ND | ND | ND | – |
| Using of L-arginine | + | + | - | + | - | - | – |
| Hydrolysis of Tween-80 | + | + | + | - | + | + | + |
| Tyrosinase production | ND | - | ND | ND | - | ND | + |
| Hydrolysis of phenyl Alanine | ND | - | ND | - | + | ND | – |
| Hydrolysis of casein | ND | + | + | ND | - | ND | + |
| Egg-yolk lecithinase | ND | + | + | ND | - | ND | + |
| Growth at pH>7 MVRP | ND | + | ND | ND | + | ND | + |
| Growth at pH<6 MVRP | ND | + | ND | ND | + | ND | + |
| Glucose fermentation | + | + | + | + | - | + | + |
| Arabinose fermentation | - | - | + | + | - | - | – |
| Xylose fermentation | - | - | - | - | - | - | – |
| Mannitol fermentation | + | - | - | + | - | - | – |
| Sucrose fermentation | + | + | + | - | - | + | + |
| Optimum pH | 6–8 | 6–8 | 6–8 | 7–8 | 6–8 | 6–8 | 6–8 |

and Mm7. Gelatine hydrolysis was positive in all isolates except Mm4 and Mm6. The isolates grew optimally in nutrient broth medium where pH values were 6–8. All of the isolates were facultatively anaerobic except Mm5. Growth was observed for all isolates in the presence of lysozyme.

Based on morphological, nutritional, physiological, and biochemical characteristics in detail, the isolates were identified as *Pseudomonas* sp. (Mm1), *Bacillus thuringiensis* (Mm2), *Pseudomonas* sp. (Mm3), *Enterobacter* sp. (Mm4), *B. sphaericus* (Mm5), *Acinetobacter* sp. (Mm6), and *B. weihenstephanensis* (Mm7) from the larvae and adults of *M. melolontha*.

Isolates 1 and 3 have gram-negative rods, are non-spore forming, facultatively aerobic, motile, do not hydrolyse starch, and produce acid from glucose. Isolates 1 and 3 showed some features of *P. fluorescens*, *P. aeruginosa*, and *P. chlororaphis*. These bacteria were thus identified as *Pseudomonas* sp. Sixteen species of genus *Pseudomonas* are found in insects. In the literature, *P. aeruginosa* and *P. fluorescens* were frequently isolated from insects (Sezen & Demirbağ 1999; Sezen et al. 2004, 2005).

Isolate 2 is rod-shaped, motile, has gram-positive rods, 0.85–1.04 × 3.13–4.75 μm, is catalase positive, spore-forming, produces acid from glucose, reduces nitrate, and has cream colonies with a dry, corrugated surface on nutrient agar, elipsoid-central spores, and parasporal crystals. Morphological, physiological, and biochemical tests showed that this bacterium is *Bacillus thuringiensis* (Bucher 1981; Sneath 1986; Thiery & Frachon 1997). Recently this bacterium has been isolated from other hazelnut pests, *Balaninus nucum* (Sezen & Demirbağ 1999) and *Malacosoma neustria* (Kati et al. 2005).

Isolate 4 is motile, nonspore-forming, has gram-negative rods, 0.66–0.85 × 0.95–1.9 μm, and is catalase positive. Isolate 4 showed some features of different *Enterobacter*. This isolate was identified as *Enterobacter* sp. Up to now, various bacteria belonging to Enterobacteriaceae have been isolated and identified from different insects (Lipa & Wiland 1972; Bucher 1981; Sezen & Demirbağ 1999; Sezen et al. 2004).

Isolate 5 has gram-positive rods, is facultatively aerobic, catalase and oxidase positive, does not hydrolyse urea or casein, does not use citrate or L-arginine, reduces nitrate, hydrolyses phenyl alanine, and does not produce egg-yolk lecithinase. The bacterium was identified as *B. sphaericus*. Recently, it has been isolated from other hazelnut pest, *Anoplus roboris* (Demir et al. 2002).

Isolate 6 is motile, nonspore-forming, has gram-negative rods, 0.76–0.85 × 1.04–1.42 μm and does not reduce nitrate. Morphological, physiological, and biochemical tests showed that isolate 6 is *Acinetobacter* sp. *Acinetobacter* is very common in insects and widely distributed in nature and is normally found in soil and water (Bucher 1981).

Isolate 7 is rod-shaped, motile, has gram-positive rods, 0.85–0.95 × 2.85–7.6 μm, is catalase positive, spore-forming, produces acid from glucose, reduces nitrate, has cream and filamentous colonies after 1 day of incubation on nutrient agar plate at 30°C. Morphological, physiological, and biochemical tests showed that this bacterium is *B. weihenstephanensis* (Lechner et al. 1998).

We determined the number of bacteria in larvae and adults by counting the number of colonies on the plates which were inoculated with diluted bacterial suspensions. The total numbers of bacteria were found to be $4.4 \times 10^6 \pm 0.325$ cfu/larva ($n = 20$) and $2.04 \times 10^7 \pm 0.310$ cfu/adult ($n = 20$) in *M. melolontha*.

We recorded that larvae showed general symptoms before death following 10 days of infection with the bacterial isolates; the larvae displayed sluggishness and loss of appetite. In addition to the other major symptoms, infection decreased larval survival. The average duration of larval life span of healthy individuals at 26 ± 2°C was 60 ± 2 days, whereas the average life span for infected larvae at 26 ± 2°C was 10 ± 3 days.

The insecticidal activities of the bacterial isolates and crystal proteins on the larvae of *M. melolontha* are shown in Fig. 1. In all bioassays, the highest insecticidal activity of the bacterial isolates on the larvae of *M. melolontha* was 80% with *B. thuringiensis* (Mm2) and *B. weihenstephanensis* (Mm7) within 10 days (ANOVA LSD, $P < 0.05$). The activities of the rest of the samples were 40% with *Pseudomonas* sp. (Mm1), 50% with *Pseudomonas* sp. (Mm3), 20% with *Enterobacter* sp. (Mm4), 60% with *B. sphaericus* (Mm5), and 0% with *Acinetobacter* sp. (Mm6) on the larvae of *M. melolontha* within 10 days. Sezen & Demirbağ (1999) observed that the bacterial isolates from *Balaninus nucum* (Coleoptera: Curculionidae) have differential insecticidal effects on the *B. nucum* larvae. In our study, *B. thuringiensis* isolated from *M. melolontha* has very high insecticidal effects on *M. melolontha* larvae (80%). In another study, it was determined that this isolate contained *cry*3

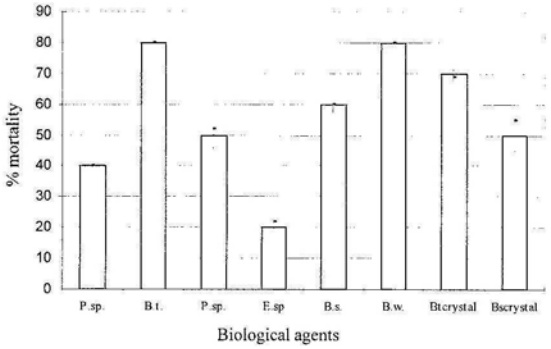


**Fig. 1** Results of insecticidal effects of bacterial isolates and crystals on *Melolontha melolontha* larvae 10 days after exposure. Bacterial isolates: P.sp., *Pseudomonas* sp. (Mm1); B.t., *Bacillus thuringiensis* (Mm2); P.sp., *Pseudomonas* sp. (Mm3); E.sp., *Enterobacter* sp. (Mm4); B.s., *Bacillus sphaericus* (Mm5); B.w., *Bacillus weihenstephanensis* (Mm7); Btcrystal, *Bacillus thuringiensis* crystal protein; Bscrystal, *Bacillus sphaericus* crystal protein. Vertical bars represent standard deviation. ANOVA ($F$= 52.678; $P < 0.05$) LSD test.

gene, specific for coleopteran species (Kurt et al. 2005).

*Bacillus thuringiensis* is now the most widely used biologically produced pest control agent. Worldwide sales (1995) of *B. thuringiensis* were US$90 million, representing c. 2% of the total global insecticide market. Rowe et al. (1987) reported annual worldwide distribution of *B. thuringiensis* as $2.3 \times 10^6$ kg. In early 1998, there were nearly 200 registered *B. thuringiensis* products in the United States. Although use of biological pesticides in agriculture remains significantly behind that of synthetic chemical pesticides, several environmental and safety considerations favour the future development of *B. thuringiensis*. Cry proteins that have been studied thus far are not pathogenic to mammals, birds, amphibians, or reptiles, but are very specific to insects and invertebrate pests against which they have activity. Cry-based pesticides generally have low costs for development and registration (Crickmore et al. 1998).

*Bacillus sphaericus* showed 60% insecticidal effect on *M. melolontha* larvae. Wirth et al. (2000) observed that the Cyt1A toxin of *B. sphaericus* has very important insecticidal effects against resistant mosquito populations. In another study, a similar result was recorded, where a strain of *B. sphaericus* isolated from *Anoplus roboris* Sufr. (Coleoptera: Curculionidae) had 67% insecticidal effect on its adults (Demir et al. 2002).

In this study, we determined the insecticidal effect of two *Pseudomonas* species on *M. melolontha* larvae. Many scientists have isolated bacteria that belong to the *Pseudomonas* genus and especially *P. fluorescens* from various insects (Lipa & Wiland 1972; Sezen & Demirbağ 1999). In addition, *P. aeruginosa* (Osborn et al. 2002) and *P. chlororaphis* (Bucher 1981) were isolated and characterised from various pests.

The insecticidal activities were determined as 20% and 0% with *Enterobacter* sp. and *Acinetobacter* sp. on the larvae of *M. melolontha*, respectively. *Enterobacter* and *Acinetobacter* are very common in insects and widely distributed in nature and normally found in soil and water (Bucher 1981).

Crystal proteins of *Bacillus* sp. had lower insecticidal activity on *M. melolontha* larvae. The highest insecticidal activity was 70% with Mm2 within 10 days. The insecticidal activity of *B. sphaericus* was 50% within 10 days. Lopez-Meza & Ibarra (1996) recorded that parasporal crystals of *B. thuringiensis* isolated from living larvae of *Anopheles pseudopunctipennis* had no toxicity against four species of caterpillar, three species of mosquito, and two species of beetle.

Consequently, it was determined that *B. weihenstephanensis* and *B. thuringiensis* in particular, and the crystal proteins of *B. thuringiensis*, can be used as a biological control agent against *M. melolontha* larvae. Future studies will be conducted with the aim of finding a biological control agent against this hazardous insect using this pesticide or other newly improved pesticides. The present study has contributed significantly to the literature on bacterial isolates and biological control of the common cockchafer.

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