Turk J Biol
35 (2011) 275-282

doi:10.3906/biy-0902-19

An entomopathogenic bacterium, *Pseudomonas putida*, from *Leptinotarsa decemlineata*

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Received: 18.02.2009

Abstract: The Colorado Potato Beetle (CPB), *Leptinotarsa decemlineata* (Say) (Col.: Chrysomelidae), is a pre-eminent pest on solanaceous crops worldwide, and much research has focused on the different components of its variable population dynamics. *L. decemlineata* has developed resistance to insecticides used for its control. Consequently, alternative methods are needed urgently for the control of this pest. In this study, in order to find a more effective and safer biological control agent against *L. decemlineata*, we isolated a new bacterial isolate (Ld4) from *L. decemlineata*, and tested it for insecticidal effects on *L. decemlineata* larvae. Also, the insecticidal effect of Ld4 was compared with Ld3, which was isolated from *L. decemlineata*. According to the morphological, physiological, biochemical tests, API20E and API50CH panel test systems, and 16S rRNA sequence homologies, this isolate was identified as *Pseudomonas putida*. The highest insecticidal effect determined on *L. decemlineata* within 5 days was 100%, and this activity was exhibited by Ld4 isolate. Our results indicate that Ld4 isolate may be valuable as a biological control agent for the biological control of *L. decemlineata*.

Key words: *Pseudomonas putida*, insecticidal activity, microbial control

Leptinotarsa decemlineata'dan izole edilen bir entomopatojenik bakteri, *Pseudomonas putida*

Özet: Patates böceği, *Leptinotarsa decemlineata* (Say) (Col.: Chrysomelidae), solanaceous ürünler üzerinde dünya çapında önemli bir zararlıdır ve çok sayıda araştırma çeşitli populasyon dinamiklerinin farklı bileşenleri üzerine yoğunlaşmıştır. Patates böceği, kontrolü için kullanılan, insektisidlere aşırı dirençlilik geliştiren bir türdür. Zararlının kontrolü için acilen farklı metotlara ihtiyaç vardır. Bu çalışmada, *L. decemlineata*'ya daha etkili ve daha güvenilir bir kontrol ajanı bulabilmek için, patates böceğinden yeni bir bakteriyal izolat (Ld4) izole edildi ve zararlı larvaları üzerindeki insektisidal etki testleri yapıldı. Ld4'ün insektisidal etkisi, *L. decemlineata*'dan izole edilen Ld3 ile karşılaştırıldı. Morfolojik, fizyolojik, biyokimyasal, API20E ve API50CH panel test sistemleri ve 16S rRNA gen dizi analizi sonuçlarına göre, bu yeni izolat *Pseudomonas putida* olarak tanımlandı. Ld4 izolatı beş günlük süreçte patates böceği üzerinde %100'lük bir etki gösterdi. Elde edilen sonuçlar gösterdi ki Ld4 izolatı *L. decemlineata*'nın biyolojik kontrolü için önemli bir biyolojik kontrol ajanı olabilir.

Anahtar sözcükler: *Pseudomonas putida,* insektisidal aktivite, mikrobiyal kontrol

Introduction

Potato is an important crop, whose production amounts to 4.3 million tons on 192,000 hectares of growing area in Turkey (1). The Colorado Potato Beetle (CPB), *Leptinotarsa decemlineata* (Say), is one of the most important pests, and many methods have been used to control it, including hand-picking, bird predation, introduction of natural enemies, trapping border sprays, trench traps, propane flames, and crop rotation (2). Both adults and larvae feed on and often cause complete defoliation of the potato plants attacked, with considerable yield losses (50% of the crop in some EPPO countries) (3). Genetically engineered resistant varieties containing toxin genes from *Bacillus thuringiensis tenebrionis* are highly effective, but they are not now used because of marketing concerns and availability of limited number of transgenic varieties. Also, recombinant defense molecules in plants may affect parasitoids or predators indirectly (4).

In general, the control of this pest is accomplished by utilizing insecticides. However, during the 1980s, *L. decemlineata* began to develop resistance against many insecticides commonly used for its control. This insect is well known for its rapid resistance development to pesticides, recently including imidacloprid, a neonicotinoid compound (5). Also, the natural enemies of the insect were influenced by pesticides used against the *L. decemlineata* (6). However, the development of insecticide resistance in target populations and concern about the detrimental effects of these chemicals on non-target arthropods, the environment, and human health have spurred interest in alternative insect control agents.

At present, many pesticides are used in Turkey to control *L. decemlineata*, including endosulfan, deltametrin, chlorpyrifos-ethyl, azinphos, promecarb, and cypermethrin. Increasing interest in developing environmentally safe pest control methods has inspired us to study the potential of bacteria for controlling *L. decemlineata*. Because there is no study on the investigation of entomopathogenic bacteria as biological control agent of *L. decemlineata*, this insect is very interesting for biological control researchers. Infected insect larvae present certain symptoms that suggest bacterial infection; they become flaccid, lethargic, and stop eating (7).

It is known that many bacteria that can be isolated from insects belong to the families Bacillaceae, Enterobacteriaceae, and Pseudomonaceae. However, bacteria-insects interactions are not only pathogenic but also symbiotic. Symbiotic bacteria are ubiquitously located in the insect's guts with these symbioses ranging from pathogenic to mutualistic and from facultative to obligate (8). Various bacterial insect pathogens are used successfully in microbial control of insects (9-14).

"Pseudomonad" is a general term for a diverse group of bacteria that morphologically and physiologically resemble members of the genus *Pseudomonas*. They share the following characteristics: Gram-negative, rod-shaped, non-spore forming, typically motile with one or more polar flagella, aerobic metabolism, and motile by means of a single polar flagellum (15).

Most pseudomonas are free-living saprophytic organisms in soil or water where they play an important role in decomposition, biodegradation, and the carbon and nitrogen cycles. Because of this lifestyle, pseudomonas are characterized by great metabolic diversity and are able to utilize a wide range of carbon sources, including molecules that few other organisms can break down (16,17).

Since Ld4 has strong insecticidal activity against the larvae of *L. decemlineata* among the bacterial isolates from *L. decemlineata*, we decided to study this strain. Here, we reported the isolation and identification of the bacterium found in living *L. decemlineata* larvae. We also tested the insecticidal activity of this bacterial isolate against the larvae of *L. decemlineata* as a possible biological control agent.

Material and methods

Collection of insects

The larvae of *L. decemlineata* were collected from areas of potato fields in the Trabzon, Turkey, during the period of 2005-2006 in the morning and at sunset in May and June. Collected insects were brought in a container proving comfortable conditions for air and light to the Microbiology Laboratory at the Department of Biology, Karadeniz Technical University, Trabzon, Turkey.

Isolation and identification of bacterium from *L. decemlineata*

Insect larvae were examined macroscopically, and the surface of healthy larvae was sterilized in 70% alcohol to remove possible contaminants (15,18). The larvae were homogenized in a nutrient broth by using a glass tissue grinder. The suspension was filtered twice through 2 layers of sterilized cheesecloth to remove the debris (18). The suspension from larvae was diluted to 10^{-8} (19). One hundred microliters of each suspension was plated on a nutrient agar. Plates were incubated at 28 °C for 24-48 h. After colonies were obtained, according to the colour and morphology of the colonies, the bacteria were separated, and then pure cultures of colonies were prepared. They were named as Ld1-6. Because of the pathogenicity of one of them, Ld4, it had very insecticidal activity on the larvae, this paper focussed on this bacteria. Also, the other isolate, Ld3, was used as non-pathogenic bacterium for pathogenity experiments. These cultures, Ld3 and Ld4, were identified by various tests. Tests such as utilization of organic compounds, spore formation, NaCl tolerance, optimum temperature, catalase test, oxidase test, and gelatine hydrolysis were performed for this isolate. The identification procedure of isolated bacteria was done according to "Bergey's Manual of Systematic Bacteriology 1 and 2" (20,21).

API 20E and API 50CH panel test systems

For further identification, API 20E and API 50CH strips were used. These tests, API 20E and API 50CH, are standardized systems, associating respectively 20 and 50 biochemical tests for the study of the carbohydrate metabolism of microorganisms. These tests were performed according to the procedure suggested by Alsina and Blanch (20) with some modifications. Bacterial colony of the isolate was diluted in 0.85% NaCl solution. The amount of bacteria was adjusted to $1 \times 10^{15\text{-}16}$ cfu/mL. Two hundred microliters of this solution were transferred into each well of the test systems. To prevent any contact with air, the wells were filled up with mineral oil. Then the panels were incubated for 18-24 h at 30°C. API 20E and API 50CH systems were used only to characterize the bacterial isolate, because, as pointed out by Behrendt et al. (21) and Peix et al. (22), the identification of non-clinical isolates is often wrong with these systems.

16S rDNA sequencing

For 16S rDNA sequencing, DNA was extracted as described previously (23). Amplification and sequencing of the nearly complete 16S rDNA gene was performed according to conventional methods. PCR amplification was done by using UNI Primers. Specific primers for identification of 16S rDNA (5'- ATT CTA GAG TTT GAT CAT GGC TCA-3'; reverse, 5'- ATG GTA CCG TGT GAC GGG CGG TGT GTA-3') were synthesized and used in the PCR. Each experiment was associated with negative (without DNA template) controls. The PCR product was cloned into pGEM-T Easy Vector (Promega) and transformed to *Escherichia coli* JM101 strain. Sequencing of the 16S rDNA genes had been performed with an Applied Biosystems model 373A DNA sequencer by Macrogen Inc. (Seoul, Republic of Korea). A sequence consisting of about 1400 nt of the 16S rDNA gene of isolates was determined. The sequence obtained was compared with those from GenBank using the BLAST program (24) and 16S rDNA gene sequence of Ld4 has been deposited in GenBank under accession number GU187010.

Growing conditions for strains Ld3 and Ld4

Bacteria were incubated in a nutrient broth medium at 30 °C for 18 h. After incubation, the bacterium density was measured as 1.89 at OD_{600} (1.8×10^9 cfu/mL) (25-27). Five milliliters of these cultures were centrifuged at 3000 rpm for 10 min. The pellets were re-suspended in 5 mL of sterilized PBS and used in bioassays.

General conditions for experimental infections

A diet was prepared from the pieces of leaves (2-3 cm^2) of *Solanaceae* sp. The leaves were placed into individual sterilized glass containers (80 mm in diameter). The suspension of bacterial isolate adjusted as (1.8×10^9 cfu/mL) prepared in PBS was applied equally to the surface of the 2 leaves. Then 10 second and third instar healthy larvae were placed on the diet in containers, and fed with the leaves included bacterial isolate. Thus, each of the 10 larvae were fed with the bacterial culture adjusted as 1.8×10^8 cfu/mL. The containers were kept at 26 ± 2 °C and 60% room humidity on a 12:12 h photo regime, with the diet changed after eating. The mortalities of

larvae were recorded every 24 h and all dead larvae were removed from containers. Infectivity tests were carried out, with the untreated controls (with PBS). At least 30 larvae were assayed for Ld3 and Ld4 isolates. Bioassays were repeated 3 times on different occasions. Means were analyzed using one-way analysis of variance (ANOVA) and were compared by least significant difference (LSD) test (28).

Results and discussion

There has recently been an increasing interest in finding more pathogenic for insects and safer for humans bacterial isolate against hazardous insects. Insect predators, parasitoids, and parasites such as viruses (29), rickettsia, nematodes, fungi (30), and bacteria (14) have also been identified and used for biological control in associated some insect. Although there are a lot of biological control studies on *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), to date, there has been no study on the isolation and characterization of the entomopathogenic bacteria from *L. decemlineata* as potential biological control agents. This is the first study on the isolation, identification, and insecticidal activity of entomopathogenic bacteria from *L. decemlineata*.

In this study, based on comparative analysis of 16S rDNA gene sequence and phenotypic characteristics of bacterial isolate, we reported the characterization in detail and the results of insecticidal activity of a *Pseudomonas putida* (Ld4) and *Acinetobacter* sp. (Ld3) from *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). We decided to study the further characterization of these isolates. Of these, Ld4 has a highly insecticidal activity. Therefore, we also reported the results of the insecticidal activity of these bacterial isolates (Ld3 and Ld4) on the larvae of *L. decemlineata*.

Ld3 and Ld4 are rod-shaped, Gram-negative, basil, non spore-forming, catalase positive, and form cream and round colonies on nutrient agar. They produced acid from glucose and did not reduce starch. Ld3 and Ld4 were grown at 37 °C and 40 °C. Ld3 is non-motile but Ld4 is motile (Table 1). Conventional biochemical tests are determined using the API 20E and API 50CH strips (bioMerieux) (Table 2, Table 3).

Table 1. The morphological, physiological, and biochemical characteristics of Ld3 and Ld4 from *L. decemlineata*.

Tests	**Ld3**	**Ld4**
Colony color	Cream	Cream
Shape of bacteria	Rod	Rod
Gram stain	–	–
Spore stain	–	–
Motility	–	–
Catalase	+	+
Hydrolysis of starch	–	–
Oxidase	–	+
Hydrolysis of urea	+	+
Citrate utilization	–	+
Indole test	–	–
Methyl red test	–	+
Voges proskauer test	–	–
Growth in 4% NaCl	+	+
Growth in 6.5% NaCl	–	–
Growth at 4 °C	–	–
Growth at 37 °C	+	+
Growth at 40 °C	+	+
Growth at 44 °C	–	–
L-Arabinose	–	–
D-Xylose	–	–
D-Glucose	+	+
L-Lactose	–	–
Mannitol	–	–
Rafinose	–	–
D-Ribose	–	–
Maltose	–	–
PIA	ND	+

We used a method based on the polymerase chain reaction (PCR) that allows rapid and highly sensitive determination of the 16S rRNA gene content of Ld4 isolate. The PCR result showed that Ld3 and Ld4 isolates have one fragment with the expected sizes of about 1400 bp corresponding to the 16S rDNA gene. After the sequences were obtained, they were compared with those in Genbank. The biochemical properties of the Ld3 is matched the *Acinetobacter* sp.; however, it could not identify at the species level. The genus has been known for many years to be genotypically heterogeneous (31,32). Many

Table 2. The API 20E test results of Ld4 from *L. decemlineata*.

Tests	Ld4
ONPG (2-nitrophenyl-βD-galactopyranoside)	–
ADH (L-arginine)	+
LDC (L-lysine)	–
ODC (L-ornithine)	–
CIT (trisodium citrate)	+
H_2S (sodium thiosulfate)	–
URE (urea)	–
TDA (L-tryptofane)	–
IND (L-tryptofane)	–
VP (sodium piruvate)	–
GEL (gelatine (origine bovine))	–
GLU (D-glucose)	+
MAN (D-mannitol)	–
INO (inositol)	–
SOR (D-sorbitol)	–
RHA ((L-rhamnose)	–
SAC (D-saccharose)	–
MEL (D-melibiose)	–
AMY (amygdaline)	–
ARA (L-arabinose)	–
OX	+
NO_2	–
N_2	–

taxonomists have considered this classification insufficient given the considerable heterogeneity among members of the genus. Unfortunately, there is no single biochemical test or set of tests available that enables the accurate identification of strains at the DNA group level. The biochemical properties of the Ld4 importantly differed from those that matched the species in Genbank, except *P. putida*. In addition to the results of physiological and biochemical tests, the 16S rDNA sequence of bacterial strain Ld4 showed 99% similarity to those of *P. putida*. Both *P. putida* in literature and new isolate Ld4 have positive results for tests oxidase, D-glucose, D-fructose, L-arginine, and citrate. Also, they share same negative results for D-arabinose, D-maltose, D-celiobiose, D-lactose, starch, inulin, L-rhamnose, D-saccharose, D-trehalose, erythritol, D-sorbitol, inositol, and D-adonitol (16,17).

Table 3. The API 50CH test results of Ld3 and Ld4 from *L. decemlineata*.

Tests	Ld3	Ld4
GLY (glycerol)	–	+
ERY (erythritol)	–	–
DARA(D-arabinose)	–	–
LARA(L-arabinose)	–	–
RIB (D-ribose)	–	–
DXYL (D-xylose)	–	+
LXYL (L-xylose)	+	–
ADO (D-adonitol)	–	–
MDX (methyl-βD-xylopyranoside)	–	–
GAL (D-galactose)	–	+
GLU (D-glucose)	+	+
FRU (D-fruktose)	+	+
MNE (D-mannose)	–	+
SBE (L-sorbose)	+	–
RHA (L-rhamnose)	–	–
DUL (dulcitol)	–	–
INO (inositol)	–	–
MAN (D-mannitol)	–	–
SOR (D-sorbitol)	–	–
MDM (metyl-αD-mannopyranoside)	–	–
MDG (metyl-αD-glukopyranoside	–	–
NAG (N-acetylglukosamine)	–	–
AMY (amygdaline)	–	–
ARB (arbutin)	–	–
ESC (esculin (ferric citrate))	–	–
SAL (salicin)	–	–
CEL (D-celiobiose)	–	–
MAL (D-maltose)	–	–
LAC (D-lactose (bovine origin))	–	–
MEL (D-melibiose)	–	–
SAC (D-saccharose)	–	–
TRE (D-trehalose)	–	–
INU (inulin)	–	–
MLZ (D-melezitose)	–	–
RAF (D-raffinose)	–	–
AMD (amidon (starch))	–	–
GLYG (glycogen)	–	–
XLT (xylitol)	–	–
GEN (gentiobiose)	–	–
TUR (D-turanose)	+	–
LYX (D-lyxose)	–	–
TAG (D-tagatose)	–	–
DFUC (D-fucose)	–	+
LFUC (L-fucose)	+	–
DARL (D-arabitol)	–	–
LARL (L-arabitol)	–	–
GNT (potassium glukonate)	–	+
2KG (potassium 2-ketoglukonate)	–	+
5KG (potassium 5-ketoglukonate)	–	–

Table 4. The insecticidal effect of the isolates (Ld4 and Ld3) on *L. decemlineata* larvae within 5 days. ANOVA LSD test, $P < 0.05$.

Isolate/ Days	Mortality (%)					(Mean ± SD) Total
	first	second	third	fourth	fifth	
Control (PBS)	-	5	2.5	5	2.5	15±3
Ld3	-	12.5	22.5	7.5	2.5	45 ± 5
Ld4	-	27.5	27.5	20	25	100 ± 5

Sixteen species of the genus *Pseudomonas* have been found in insects to date. Isolation of *P. aeruginosa* and *P. fluorescens* from insects has frequently been reported in literature (10,33,34). In previous studies, it was reported that this bacterium could infect *Euproctis chrysorrhoea* (Lepidoptera, Lymantriidae) (17), *Mamestra brassicae* (Lepidoptera, Noctuidae) (35), and *Dendroctonus micans* (Coleoptera: Scolytidae) (36). A number of *Pseudomonas* species are important insect pathogens, i.e. *P. aeruginosa* (7), *P. fluorescens* (10) and *P. chlororaphis* (37). Although most of the *P. putida* isolates appear insignificant as an insect pathogen (38), our *P. putida* isolate (Ld4) is highly pathogenic against *L. decemlineata* larvae. This situation can be explained by the specific strains of bacteria, not just the species.

P. putida is a rapidly growing bacterium frequently isolated from most temperate soils and waters, particularly from polluted soils. Pseudomonads engage in important metabolic activities in environments including element cycling and the degradation of biogenic and xenobiotic pollutants (39), and also they can be found in the intestines of animals and on the surface of plant materials. Intestinal bacteria are able to play a key role in the nutritional physiology of their hosts such as producing short-chain fatty acids from carbohydrates or synthesizing amino acids (40). The midgut bacteria could be parasites living in the gut, relying on the nutrients that the host enzymes have digested (41).

We also recorded that larvae showed general symptoms before death following the first day of feeding with the bacterial isolate; the larvae displayed sluggishness and loss of appetite. Infected insect larvae present certain symptoms that suggest bacterial infection; they become flaccid, lethargic, and stop eating (7). In addition to other major symptoms, infection decreased larval survival. The average duration of larval life span of healthy individuals at 26 ± 2 °C was 30 ± 2 days, whereas the average life span for infected larvae at 26 ± 2 °C was 5 ± 3 days.

We have shown evidence of the potential of Ld4 isolate for *L. decemlineata*. The insecticidal activity of the bacterial isolate (Ld4) prepared in PBS on the larvae of *L. decemlineata* is shown in Table 4. The insecticidal activity is 100% for Ld4 using 1.8×10^9 cells/mL at OD_{600} on *L. decemlineata* larvae within 5 days (ANOVA LSD, $P < 0.05$). The capacity of this strain to control *L. decemlineata* is clear. The insecticidal activity of bacterial isolate has a strong effect against the larvae of *L. decemlineata* as possible biological control agents.

Consequently, it was determined that *P. putida* (Ld4) may be valuable as a microbial control agent for coleopteran insects. Future studies will be conducted with the aim of finding a better commercial microbial control agent against this pest using this or other newly improved biological materials. The present study has contributed significantly to the literature on bacterial isolates and microbial control of the *L. decemlineata*.

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