View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by AJOL - African Journals Online

African Journal of Biotechnology Vol. 8 (24), pp. 7111-7115, 15 December, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

High insecticidal activity of *Leclercia adecarboxylata* isolated from *Leptinotarsa decemlineata* (Col.: Chrysomelidae)

Hacer Muratoglu, Hatice Kati, Zihni Demirbag and Kazım Sezen*

Karadeniz Technical University, Faculty of Arts and Sciences, Department of Biology, 61080 Trabzon, Turkey.

Accepted 14 April, 2009

Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is an important pest on solanaceous crops worldwide. CPB has developed resistance to insecticides used for its control. In this study, in order to find a more effective and safer biological control agent against *L. decemlineata*, we studied the bacterial flora of CPB, and tested them for insecticidal effects on it. The highest insecticidal effect determined on *L. decemlineata* within 5 days was 100% and this effect was exhibited by Ld1 isolate. According to the morphological, physiological and biochemical tests, and 16S rRNA sequence homologies, Ld1 was identified as *Leclercia adecarboxylata*. This is the first time that this bacterium has been isolated from any insect pests. Our results indicate that *Lecl. adecarboxylata* may be valuable as a biological control agent for *L. decemlineata.*

Key words: Insecticidal activity, *Leclercia adecarboxylata*, *Leptinotarsa decemlineata*, microbial control.

INTRODUCTION

Potato is an important crop and Turkey produces 4.3 million tons of it on 192,000 ha of growing area in Turkey (Anonymous, 2000). *Leptinotarsa decemlineata* is one of the most widespread and destructive pests of potato. Both adults and larvae feed on this host and often cause complete defoliation of the potato plants attacked, with considerable yield losses (50% of the crop in some EPPO countries). *L. decemlineata* is also suspected of spreading several potato diseases, including *Ralstonia solanacearum* and *Clavibacter michiganensis* subsp. *sepedonicus* (URL-1).

Many methods have been used to control CPB, including hand-picking, bird predation, introduction of natural enemies, trapping border sprays, trench traps, propane flames and crop rotation (Casagrande, 1987). Genetically engineered resistant varieties containing toxin genes from *Bacillus thuringiensis* var. *tenebrionis* are highly effective (Reed et al., 2001), but they are not now used because of marketing concerns and limited number of transgenic varieties available. Also, recombinant defense molecules in plants may affect parasitoids or predators indirectly (Bouchard et al., 2003).

In general, the control of this pest is accomplished by utilizing insecticides. However, during the 1980's, resistance against many insecticides commonly used for its control began to develop. This insect is well known for developing rapid resistance to pesticides, recently including organophosphates, carbamates and pyrethroids (Forgash, 1985). The CPB is ranked among the top 10 most resistant species to insecticides in the world (Mota-Sanchez et al, 2002). Also, the natural enemies of the insect were influenced by pesticides used against the CPB (Ferro and Boiteau, 1993).

Leclerciaes are distributed widely in nature and have been isolated from food, water and other environmental sources. *Lecl. adecarboxylata* share the characteristics of typical (Richard, 1989; Tamura et al., 1986; Teramoto and Sakazaki, 1984) and also isolated from various clinical specimens, including blood, faces, sputum, urine and wound pus (Richard, 1989; Tamura et al., 1986). It has not yet been reported that this species has been isolated from any microbial flora of insects.

Microscopic techniques, physiological and biochemical tests generally cannot distinguish morphologically similar but metabolically different bacteria. Relatedness of species could be determined with the arrival of molecular

*Corresponding author. E-mail: sezen@ktu.edu.tr.

techniques in bacterial taxonomy. Nucleic acid pairing studies on entire genomes or selected genes is the basis for comparison between species. Similarly, the potential of sequence analysis of gene coding for rRNA and of certain proteins for interfering with the evolution of taxa through billions is documented (Woese, 1987). The introduction of PCR amplification of 16S rRNA genes from bacterial DNA is used to differentiate the bacteria from each other. The primary structure of the 16S rRNA is highly conserved and species having 70% or greater DNA similarity usually have more than 97% sequence identity (Stackebrandt et al., 1992).

In this study, in order to find and identify a new toxic bacterial isolate against *L. decemlineata*, we isolated the entomopathogenic bacteria from *L. decemlineata*, characterized in detail and tested for the insecticidal activity.

MATERIALS AND METHODS

Collection of insects

Adults and larvae of *L. decemlineata* were collected from potato fields in Trabzon, Turkey, during the period of 2005 - 2007. Collected insects were brought to the microbiology laboratory in the department of biology Karadeniz technical university, Trabzon, Turkey.

Isolation and identification of bacteria from *L. decemlineata*

The insects examined macroscopically were distinguished as dead, diseased and healty and the surface was sterilized in 70% alcohol to remove possible contaminants (Lipa and Wiland 1972; Poinar and Thomas, 1978). The larvae were homogenized in a nutrient broth by using a glass tissue grinder. The suspension was filtered twice through a 2-layered cheesecloth to remove debris (Poinar and Thomas, 1978). Then, it was diluted to 10^{-8} (Christine and Ted, 1992). 100 µl of each suspension was plated on a nutrient agar. Plates were incubated at 30°C for 24 - 48 h. According to the colour and morphology of the colonies, the bacteria were separated. Then, the pure cultures of colonies were prepared and these cultures were identified by various tests. Tests such as utilization of organic compounds, spore formation, NaCl tolerance, optimum temperature, lysine decarboxylase production, catalase test, oxidase test and citrate test were performed for all isolates. For further identification, API 20E and API 50CH strips were used. In addition, *E. coli* ATCC 25922 was used as positive control organism.

DNA templates and PCR analysis

For 16S rRNA sequencing, DNA was extracted as described previously (Sambrook et al., 1989). PCR amplification of 16S rRNA genes was performed with oligonucleotide primers. Reactions were routinely carried out in 50 µl. 1 µl of template DNA was mixed with reaction buffer, 150 µM (each) with deoxynucleoside triphosphate, 0.5 µM (each) with primer and 0.5 U with Taq DNA polymerase. Nearly full-length sequence was amplified with primer UNI R (5'-ATG GTA CCG TGT GAC GGG CGG TGT GTA-3') and primer UNI F (5'- ATT CTA GAG TTT GAT CAT GGC TCA-3') by using thermal cycler (Hybaid). Amplification was performed with 30 cycle program (each cycle consisting of denaturation at 94°C for 60 s, annealing at 56°C for 60 s and extension at 72°C for 120 s), followed by a final extension step at 72°C for 5 min. Each experiment was associated with negative (without DNA template) controls. PCR products were analyzed on a 1.2% agarose gel electrophoresis. Then the gel was examined in BioDoc analyse system. These fragments were cloned to pGEM-T Easy Vector (Promega) and transformed to *Escherichia coli* JM101 strain. Sequencing of the 16S rRNA genes was performed by macrogen. The sequence obtained was compared with those from GenBank using the BLAST program (Altschul et al., 1990).

Preparation of bacterial isolate for bioassay

Bacterium was 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 x 10^9 cfu/ml) (Ben-Dov et al., 1995; Moar et al., 1995). 5 ml of this culture was centrifuged at 3,000 rpm for 10 min. The pellet was re-suspended in 5 ml of sterilized PBS and used in bioassays.

General conditions for experimental infections

Bioassays were performed with coleopteran larvae *L. decemlineata* (Chrysomelidae). A diet was prepared from the leaves of *Solanaceae* sp. for *L. decemlineata*. The diet was placed into individual sterilized glass containers (80 mm in diameter). Bacterial isolates prepared in PBS were applied to the surface of the diet. 10 s and third instars larvae were placed into each container. Containers were kept at 26 ± 2°C and 60% RH 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 daily. Infectivity tests were carried out with the untreated controls. All bioassays were repeated 7 times on different occasions. Data were evaluated using Abbott's formula (Abbott, 1925).

RESULTS AND DISCUSSION

Recently, there has been an increasing interest in finding an effective and safer biological control agent against hazardous insects. Although there are a lot of biological control studies on *L. decemlineata* to date, there has been no study on the isolation and characterization of bacteria from of *L. decemlineata* as potential biological control agents. In this study, we isolated a highly effective bacterium from *L. decemlineata*. This bacterium has not been isolated from insects before.

Based on comparative analysis of 16S rRNA gene sequence and phenotypic characteristics of bacterial isolate, we reported the characterization in detail and the results of insecticidal activity of a *Lecl. adecarboxylata* isolate (Ld1) from *L. decemlineata* (Say) (Coleoptera: Chrysomelidae). This isolate is a rod-shaped, gram-negative, bacillus, non-spore-forming, motile, catalase positive, citrate positive and forms cream and round colonies on nutrient agar. It produced acid from glucose, did not reduce starch and did not hemolize human blood on the blood agar. Gelatine was not hydrolyzed. While Ld1 grew at 37°C and 40°C, it did not grow at 44°C (Table 1). API 20E and API 50CH systems were used

Table 1. Morphological, physiological and biochemical characteristics of the Ld1 isolated from *L. decemlineata*.

Tests	Ld1	Tests	Ld1
Shape and color of colony	Cream, round	Lysine decarboxylate	-
Shape of bacterium	Rod	MCA	+
Gram staining	-	EMB	+
Catalase production	+	Citrate	+
Urease production	+	KIA*	A / A, +, -
Indole production	+	Motility	+
MR	+	Oxidase	-
VP	-	Haemolysis	-
L-Arabinose	+	Gelatinase	-
D-Xylose	+	Twen 80 hydrolisis	+
D-Glucose	+ A	**Growth properties**	
L-Lactose	+, A, G	at 37˚C	+
Mannitol	+ A	at 40˚C	+
Rafinose	+ A	at 44˚C	-
D-Ribose	+ A	at % 4 NaCl	+
Maltose	+, A, G	at % 6.5 NaCl	+
Starch hydrolisation	-	at % 8.5 NaCl	+

+ = Positive growth; - = negative growth; A = acid; G = gas.
*Bottom of tube/upper of tube.

Table 2. The results of API 20E test system of Ld1 isolated from *L. decemlineata*.

Tests	Ld1
β-galactosidase	+
Arginine dihdrolase	-
Lysine decarboxilase	-
Ornithine decarboxilase	-
Trisodium citrate	-
H_2S (sodium thiosulfate)	-
Urea	-
L-tryptophan	-
Indole	+
VP (sodium pyruvate) Test	-
Gelatinase	-
D-glucose	+
D-mannitol	+
Inositol	-
D-sorbitol	+
L-rhamnose	+
D-saccharose	+
D-melibiose	+
Amygdaline	+
L-arabinose	+
Oxidase	-
NO_2	+
N_2	-

+ = Positive growth; - = negative growth.

only to characterize the bacterial isolate, because, as pointed out by Behrendt et al. (1999) and Peix et al. (2003), the identification of non-clinical isolates is often wrong with these systems (Tables 2 and 3).

Lecl. adecarboxylata were designated initially as 'Enteric group 41' or '*Escherichia adecarboxylata*'. *Lecl. adecarboxylata* phenotypically resembles *E. coli*. Lysine decarboxylase, malonate assimilation and acid production from arabitol and cellobiose, but not from adonitol and sorbitol, allowed definitive separation of *Lecl. adecarboxylata* from *E. coli*. Based on nucleic acid and protein electrophoretic techniques, '*E. adecarboxylata*' was separated from the '*Enterobacter agglomerans*' complex (Izard et al., 1985), to which it had been assigned temporarily, and renamed as *Lecl. adecarboxylata* (Tamura et al., 1986).

We used a method that was based on the polymerase chain reaction (PCR) and that allows rapid and highly sensitive determination of the 16S rRNA gene content of Ld1 isolate. DNA amplification was carried out using universal primers (UNI F, UNI R). PCR result showed that Ld1 isolate has one fragment with the expected sizes of 1,417 bp corresponding to 16S rRNA gene. After the sequences were obtained, they were compared with those genbank. The rate of the species that shared similarity was between 97 - 100% (Table 4). The biochemical properties of the Ld1 importantly differentiated those that matched the species in genbank, except *Lecl. adecarboxylata*. Since both species share several biochemical features, *E. coli* ATCC 25922 strain was used as a positive control organism for the further tests.

Table 3. The results of API 50CH test system of Ld1 isolated from *L. decemlineata*.

Tests	Ld1	Tests	Ld1
Negative Control	-	Esculin (ferric citrate)	+
Glycerol	+	Salicin	+
Erythritol	-	D-cellobiose	+
D-arabinose	-	D-maltose	+
L-arabinose	+	D-lactose	+
D-ribose	+	D-melibiose	+
D-xylose	+	D-saccharose	+
L-xylose	-	D-trehalose	+
D-adonitol	-	Inulin	-
Methyl-β-D-xylopyranoside	-	D-melezitose	-
D-galactose	+	D-raffinose	+
D-glukose	+	Amidon (starch)	-
D-fruktose	+	Glycogen	-
D-mannose	+	Xylitol	-
L-sorbose	-	Gentiobiose	-
L-rhamnose	+	D-turanose	-
Dulcitol	+	D-lyxose	+
Inositol	+	D-tagatose	-
D-mannitol	+	D-fucose	+
D-sorbitol	+	L-fucose	-
Metyl-α-D-mannopyranoside	-	D-arabitol	-
Metyl-α-D-glukopyranoside	+	L-arabitol	-
N-acetylglucosamine	+	Potassium gluconate	+
Amygdaline	-	Potassium 2-ketogluconate	+
Arbutin	+	Potassium 5-ketogluconate	-

+ = Positive growth; - = negative growth.

Table 4. Similarty of 16S rRNA gene of Ld1 to the known bacterial sequences.

Isolate	Species those mathches to Ld1	Similarities (%)	Accession number
Ld1	*Enterobacter amnigenus*	98	AB004749
	Leclercia adecarboxylata	98	AJ277977
	Pantoea agglomerans	97	AY691543
	Citrobacter braakii	97	AF025368
	Citrobacter freundii	97	AF025365
	Enterobacter ludwigii	97	AJ853891
	Enterobacter nimipressuralis	98	Z96077
	Enterobacter hormaechei	97	AJ853889
	Citrobacter werkmanii	97	AF025373
	Enterobacter aerogenes	97	AB099402
	Enterobacter cloacae	97	AY787819
	Klebsiella ornithinolytica	97	Y17662
	Enterobacter cancerogenus	97	Z96078

In addition to the result of 16S rRNA, morphological, physiological, biochemical tests, and API systems showed that this bacterium is *Lecl. adecarboxylata* (Table 2). These results of biochemical tests are in accordance with the data of Stock et al. (2004). This bacterium has been isolated for the first time from microbial flora of pest insect, *L. decemlineata*.

In conclusion, we have showed the evidence of the potential of *Lecl. adecarboxylata* as a biological control agent isolated from *L. decemlineata*. The Ld1 isolate prepared in PBS showed toxicity against tested *L. decemlineata* using 1.8 x 10^9 at OD_{600}. The recorded mortalities were 100% for *L. decemlineata* larvae within 5 days. The capacity of this strain to control coleopteran

pest *L. decemlineata* is clear.

Our results indicate that Ld1 isolate from *L. decemlineata* in Turkey is a strain of *Lecl. adecarboxylata* with a potential of use for the control of several coleopteran pests, including insects from the chrysomelidae families. It has almost identical features with *Lecl. adecarboxylata*. Also, the insecticidal activity of this new isolate was not known before. Further study will involve the detailed characterization of novel *Lecl. adecarboxylata* strains and provide good sources for developing microbial pesticides against *L. decemlineata* and other coleopteran pests.

REFERENCES

Abbott WS (1925). A Method of Computing the Effectiveness of an Insecticide. J. Econ. Entomol. 18: 265-267.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. J. Mol. Biol. 215: 403-410.

Anonymous (2000). Agricultural Structure (Production, Price, Value). Republic of Turkey. State Institute of Statistics, Ankara, p. 588.

Behrendt U, Ulrich A, Schumann P, Erler W, Burghardt J, Seyfarth W (1999). A taxonomic study of bacteria isolated from grasses: a proposed new species *Pseudomonas graminis* sp. nov. Int. J. Syst. Bacteriol. 49: 297-308.

Ben-Dov E, Boussiba S, Zaritsky A (1995). Mosquito larvicidal activity of *E. coli* with combinations of genes from *B. thuringiensis* subsp. *israelensis.* J. Bacteriol. 177: 2851-2857.

Bouchard E, Michaud D, Cloutier C (2003). Molecular interactions between an insect predator and its herbivore prey on transgenic potato expressing a cysteine proteinase inhibitor from rice. Mol. Ecol. 12:2429-2437.

Casagrande RA (1987). The Colorado potato beetle: 125 years of mismanagement. Bull. Entomo.l Soc. Am. 33:142–150.

Christine LC, Ted RJ (1992). Laboratory experiments in microbiology. Benjamin/Cummings, California.

Ferro DN, Boiteau G (1993). Management of insect pests. In: Randall CR (ed.) Potato Health Management, APS Press, St. Paul, U.S.A., FL, pp: 103-115.

Forgash AJ (1985). Insecticide resistance in the Colorado potato beetle. Massachusetts Agric. Expt. Sta. Res. Bull. 704:33-52.

Izard D, Mergaert J, Gavini F (1985). Separation of *Escherichia adecarboxylata* from the '*Erwinia herbicola–Enterobacter agglomerans*' complex and from the other *Enterobacteriaceae* by nucleic acid and protein electrophoretic techniques. Ann. Inst. Past Microbiol. 136 (suppl. B): 151-168.

Lipa JJ, Wiland E (1972). Bacteria isolated from cutworms and their infectivitiy to *Agrotis* sp. Acta Microbiol. Pol. 4: 127-140.

Lipa JJ, Aldebis KK, Vargas-Osuna E, Caballero P, Santiago-Alvarez C, Hernandez-Crespo P (1994). Occurrence, biological activity, and host range of entomopoxvirus B from *Ocnogyna baetica* (Lepidoptera: Arctiidae). J. Invertebr. Pathol. 63: 130-134.

Moar WJ, Pusztzai-Carey M , Mack TP (1995). Toxity of purified proteins and the HD-1 strain from *Bacillus thuringiensis* againts lesser cornstalk borer (Lepidoptera: Pyralidae). J. Econ. Entomol. 88: 606-609.

Mota-Sanchez D, Bills SP, Whalon ME (2002). Arthropod resistance to pesticides: status and overview. In: Wheeler WB (ed.) Pesticides in Agriculture and the Environment, Marcel Decker, Gainesville, FL, pp: 241-272.

Peix A, Rivas R, Mateos PF, Martý´nez-Molina E, Rodrý´guez-Barrueco C, Vela´zquez E (2003). *Pseudomonas rhizosphaerae* sp. nov., a novel species that actively solubilizes phosphate *in vitro*. Int. J. Syst. Evol. Microbiol. 53: 2067-2072.

Poinar GO, Jr., Thomas GM (1978) Diagnostic manual for the identification of insect pathogens. Plenum Press, New York, p. 218.

Reed GL, Jensen AS, Riebe J, Head G, Duan JJ (2001). Transgenic Bt potato and conventional insecticides for Colorado potato beetle management: comparative efficacy and non-target impacts. Entomol. Exp. Appl. 100:89-100.

Richard C (1989). Nouvelles Enterobacteriaceae rencontre´es en bacte´riologie me´dicale: *Moellerella wisconsensis, Koserella trabulsii, Leclercia adecarboxylata, Escherichia fergusonii, Enterobacter asburiae, Rahnella aquatilis*. Ann. Biol. Clin. 47:231-236.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Stackebrandt E, Liesack W, Witt D (1992). Ribosomal RNA and rRNA sequence analyses. Gene 115, 255-260.

Stock I, Burak S, Wiedemann B (2004). Natural antimicrobial susceptibility patterns and biochemical profiles of *Leclercia adecarboxylata* strains. Clin. Microbiol. Infect. 10: 724-733.

Tamura K, Sakazaki R, Kosako Y, Yoshizaki E (1986). *Leclercia adecarboxylata* gen. nov., comb. nov., formerly known as *Escherichia adecarboxylata*. Curr. Microbiol. 13:179-184.

Teramoto T, Sakazaki R (1984). Taxonomic analysis of so-called coliform organisms isolated from foods and environmental materials [in Japanese]. J. Food Hyg. Soc. Jpn. 25: 322-328.

URL-1.http://www.eppo.org/QUARANTINE/insects/Leptinotarsa decemlineata/ LPTNDE_ds.pdf

Woese CR (1987). Bacterial evol. Microbiol. Rev. 51: 221-271.