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Identification and pathogenicity of bacteria from *Gryllotalpa gryllotalpa* L. (Orthoptera: Gryllotalpidae)

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Gryllotalpa gryllotalpa L. (Orthoptera: Gryllotalpidae)'den izole edilen bakterilerin tanımlanması ve patojenitesi

Özet: *Gryllotalpa gryllotalpa* (Orthoptera: Gryllotalpidae)'nın bakteriyel mikrobiyotası, araştırılmıştır ve *G. gryllotalpa*'nın nimflerinden 10 farklı bakteriyel aerobik tür izole edilmiştir. *G. gryllotalpa* en önemli tarım zararlılarından biridir. Bu çalışmada, bakterilerin morfolojik, fizyolojik ve biyokimyasal özelliklerini ve API 20E ve API 50CH panel test sistemleri sayesinde metabolik enzim profillerini belirledik. Ayrıca, izolatların moleküler seviyede belirlenmesi için 16S rRNA geni dizi analizi ve Hiper değişken (HV) analizleri uygulanmıştır. Bakteriyel türler, *Bacillus weihenstephanensis* (Gg1), *Serratia ureilytica* (Gg2), *Bacillus simplex* (Gg3), *Bacillus nanhaiensis* (Gg5), Bacillus sp. (Gg6), *Bacillus gibsonii* (Gg7), *Citrobacter braakii* (Gg8), *Paenibacillus* sp. (Gg9), *Enterobacter* sp. (Gg10), and *Providencia rettgeri* (Gg11) olarak belirlenmiştir. Bütün bakteriyel türler, on gün boyunca 3.6×10^9 hücre/ml dozda *G. gryllotalpa*'nın nimflerine karşı test edilmiştir. Bu sonuçlar, özellikle *B. weihenstephanensis* (Gg1) ve *Bacillus nanhaiensis* (Gg5) bakterilerinin, *G. gryllotalpa*'nın mikrobiyal mücadelesinde değerli bir etken olabileceğini göstermiştir.

Anahtar sözcükler: Bakteriyel mikrobiyota, biyolojik mücadele, HV analizi, *Gryllotalpa gryllotalpa*, böcek patolojisi, 16S rRNA geni

Abstract: The bacterial microbiota of the *Gryllotalpa gryllotalpa* (Orthoptera: Gryllotalpidae) was investigated and 10 different aerobic bacterial strains were isolated from the nymphs of *G. gryllotalpa*. It is one of the most important agricultural pests. We determined the morphological, physiological, and biochemical properties of the bacteria and metabolic enzyme profiles by API 20E and API 50CH panel test systems. Additionally 16S rRNA gene sequence and Hyper variable (HV) analyses were performed to determine the strains at the molecular level. They were identified as *Bacillus weihenstephanensis* (Gg1), *Serratia ureilytica* (Gg2), *Bacillus simplex* (Gg3), *Bacillus nanhaiensis* (Gg5), Bacillus sp. (Gg6), *Bacillus gibsonii* (Gg7), *Citrobacter braakii* (Gg8), *Paenibacillus* sp. (Gg9),

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Enterobacter sp. (Gg10), and *Providencia rettgeri* (Gg11), according to results. All these bacterial strains were tested against the nymphs of *G. gryllotalpa* at 3.6×10^9 cfu/ml dose, within ten days. Our results indicate that especially *B. weihenstephanensis* (Gg1) and *Bacillus nanhaiensis* (Gg5) may be valuable as a microbial control agent for *G. gryllotalpa*.

Key words: Bacterial microbiota, biological control, HV analyses, *Gryllotalpa gryllotalpa*, insect pathology, 16S rRNA gene.

Introduction

Mole crickets in the family of Gryllotalpidae are distributed throughout temperate and tropical regions. These insects are best known for their digging forelimbs and singing from specialized burrows in the soil (Bennet-Clark 1970; 1987; Daws Bennet-Clark & Fletcher 1996). There are basically three kinds of mole crickets: two-three and the four clawed mole. And the four clawed mole crickets are distributed worldwide, with 35 species assigned to three genera, *Gryllotalpella*, *Neocurtilla* and *Gryllotalpa* (Tindale 1928).

The European mole cricket, *Gryllotalpa gryllotalpa* (Orthoptera: Gryllotalpidae) L., was introduced from Europe into the United States (Weiss 1916). European mole cricket spent nearly all their lives underground. They damage vegetable gardens, seedling beds, eat seed, cereals, potato and almost all vegetables. Shoots and young plants often perish after damage (Kobakhidze 1960; Klechkovskii 1967; Zhantiev 1991; Aydemir 2008; Baykal 2008). The insect damages on tobacco, all vegetables, corn, sunflower, cotton, fruits and forest trees in Turkey. *G. gryllotalpa* open gallery in soil and eat everything on their way while opening the gallery (Aydemir 2008). *G. gryllotalpa* is widespread in Europe, Russia, Turkey, Central Asia, Iran, Afghanistan, central and southern Asia, North Africa, America and southern Ukraine (Klechkovskii 1967).

Chlorpyripos-ethyl and parathion-methyl have been used for the control of *G. gryllotalpa* in Turkey (URL-1). Because of hazards of the chemicals in the environment, these are no longer recommended for agricultural pest management. Increasing interest in developing environmentally safe pest control methods has inspired us to study the potential microbial agents for the control of *G. gryllotalpa*.

This insect is very attractive for microbial studies. Up to now, there is no study on the investigation of the cultivable bacteria of European mole cricket (*G. gryllotalpa*). However various bacterial insect pathogens are successfully used in microbial control of the other insects (Thiery & Frachon 1997; Sezen et al. 2001; Sezen et al. 2004; 2005; 2007; Sezen et al. 2008a; Sezen et al. 2008b; Katı et al. 2009).

Bacterial insecticides, e.g. *Bacillus thuringiensis* (Bt), are the most promising alternatives. Sporulating cells of *B. thuringiensis* synthesize parasporal inclusions comprised of one or more insecticidal proteins, referred to commonly as delta-

endotoxins or insecticidal crystal proteins (Höfte & Whiteley 1989; Katı et al. 2005; Kurt et al. 2005).

It is known that many bacteria which 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 insect's guts with these symbioses ranging from pathogenic to mutualistic and from facultative to obligate (Lau et al. 2002).

Bacterial symbionts are thought to enable their hosts to survive on restrictive diets by providing nutritional supplements such as amino acids and vitamins (Buchner 1965; Baumann et al. 1995; Blattner et al. 1997). The potential use of these organisms for biological control of insect pests has driven much of the current research on bacterial symbionts. Chagas disease, for example, is a vector-borne disease that affects 16-18 million people in regions of South and Central America (Beard et al. 1992; Beard et al. 1998). The Chagas disease vector, *Rhodnius prolixus*, harbors the symbiotic bacteria *Rhodococcus rhodnii*. Beard et al. (1992; 1998) found that the symbiotic bacteria could be genetically transformed to express an antitrypanosomal agent in the gut. This discovery provides proof of principle for the use of symbionts as biological control agents. Beetles also minimize overcrowding by oxidizing aggregation pheromones into antiaggregants, both through their own and their microbial symbionts' biosynthetic pathways (Brand et al. 1975). The determination of the bacterial flora of important pest insects is an important first step in the process of using bacteria in biological control.

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 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, but no standardized guidelines exist for defining a bacterial species based on 16S rDNA (Drancourt et al. 2000), although Stackebrandt and Goebel (1994) have suggested that less than 97% 16S identity definitively denotes separate species.

More recently, sequencing of the 5' end hyper variable regions (HV) of the 16S rDNA is a useful marker for rapid and convenient identification and grouping of *Bacillus* and *Paenibacillus* species (Goto et al. 2000; 2002a). In members of the genus *Bacillus* and *Paenibacillus*, the HV region sequence is highly conserved within a species and has diverged sufficiently different between species, enabling identification and grouping of *Bacillus* and *Paenibacillus* species by sequence comparisons of the HV region, as it has shown for the genera *Brevibacillus* and *Alicyclobacillus* (Goto et al. 2000; 2002a; 2002b; 2002c; 2004; Allan et al. 2005).

Here, we report on the isolation and identification of bacteria from *G. gryllotalpa*. We also tested the insecticidal activities of these bacterial isolates against the nymphs of *G. gryllotalpa* as possible microbial control agents. This is the first study on the isolation and characterization of the culturable aerobically bacteria of *G. gryllotalpa* and for the determination of the insecticidal potential of these isolates on *G. gryllotalpa*.

Materials and methods

Collection of samples

Nymphs and adults of the European mole cricket were collected from the vicinity of Trabzon and Tokat in Turkey during the period of 2009-2011. Collected insects were brought to the Microbiology Laboratory at Department of Biology, Karadeniz Technical University, Trabzon/Turkey in sterilized bottles. Infected insect presented certain symptoms that suggest bacterial infection; they become flaccid, lethargic and stop eating.

Isolation and identification of bacteria from nymphs

Live and diseased nymphs of field collected *G. gryllotalpa* were surface sterilized with 70% alcohol for 1 min and homogenized individually in a homogenizer containing 5 ml of sterilized phosphate buffer solution (PBS, pH 7.4). Using standard aseptic techniques, surface-sterilized nymphs were individually dissected in sterile distilled water (Dugas et al. 2001). A series of dilution from 10^{-1} to 10^{-8} were prepared. One hundred µl of each suspension was plated on nutrient agar. Plates were incubated at 30°C for 24 h. For the isolation of the spore forming bacteria the hemolymph from each nymph and the suspension was obtained from homogenization were heated at 80°C for 10 min in a water bath to eliminate the non-spore-forming bacteria (Ohba & Aizawa 1986; Lee et al. 1992; Thiery & Frachon 1997). One hundred µl of the heat-treated and untreated samples were spread on a nutrient agar plates and incubated at 30°C for 48 h to 96 h (Lee et al. 1995). At the end of incubation period, the plates were examined, and bacterial colonies were selected based on color, shape and morphology of colonies. Then pure cultures of bacterial colonies were prepared, and these cultures were identified by various tests (Gram staining, spore staining, catalase-, oxidase-, indol-, VP-, citrate-, gelatin-, nitrate-, and urease-activity). The identification of isolated bacteria was done according to "Bergey's Manual of Systematic Bacteriology volume 1 and 2" (Krieg & Holt 1986; Sneath et al. 1986), API panel test systems and molecular characterizations by16S rRNA and HV analyses.

API20E panel test system

API20E (bioMérieux Inc.) biochemical test system was used for the characterization of bacteria (Kuzina et al. 2001). This test is a standardized identification system for *Enterobacteriaceae* and other non-sensitive, Gram

negative, rods which uses 21 miniaturized biochemical tests and a database. The tests were done according to the manufacturer's instructions.

API 50CH panel test system

For further identification, API 50CH strips were used. These tests are standardized systems, associating to 50 biochemical tests for the study of the carbohydrate metabolism of microorganisms. They were performed based on the method described in Alsina and Blanch (1994) with some modifications. The tests were carried out according to the manufacturer's instructions.

API 20E and API 50CH systems were used only to characterize the bacterial strains (Behrendt et al. 1999; Peix et al. 2003).

Sequence analysis of 16S rRNA genes of bacterial strains

For 16S rRNA gene sequencing, DNA was extracted from both Gram + and Gram - the bacterial strains as described previously (Sambrook et al. 1989). DNA pellets were re-suspended in 10 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4ºC until use. The amplification and sequencing of the nearly complete 16S rRNA gene was performed according to the conventional methods. PCR amplification of 16S rRNA genes of bacterial strains was performed with the following universal primers; UNI16S-L: 5'-ATT CTA GAG TTT GAT CAT GGC TCA-3'; UNI16S-R: 5'-ATG GTA CCG TGT GTG ACG G-3'. PCR amplification was performed by using BioRad Thermal Cycler. PCR reactions and amplification were carried out beginning denaturation, 2 min at 95 °C; denaturation, 1 min at 94 °C; annealing, 1 min at 56 °C; extension 2 min at 72 °C for 35 cycles; and last annealing 5 min at 72 °C. PCR products were analyzed by electrophoresis in 1% agarose gel. The gel was then examined in a BioDoc Analyse System (Biometra GmbH, Goettingen, Germany). Amplified 16S rRNA gene fragments were cloned into pGEM-T Easy Vector (Promega) and transformed to *Escherichia coli* DH10β strain. Sequencing of the 16S rRNA genes were performed by Macrogen Inc. (Seoul, Republic of Korea). So, the sequences obtained were used to perform BLAST searches (Altschul et al. 1990) using the NCBI GenBank database. Comparison of approximately 1,400 bp fragments of 16S rRNA gene sequences of each strains with other 16S rRNA sequences in the NCBI GenBank database (Altschul et al. 1990) were performed and after comparison, species that shared a similarity between 97-100% were recorded for further identification. Also, 16S rRNA gene sequences of Gg bacterial strains, have been deposited in GenBank under the accession numbers JN020634 (Gg1), JN020635 (Gg2), JN020636 (Gg3), JN020638 (Gg5), JN020639 (Gg6), JN020640 (Gg7), JN020641 (Gg8), JN020642 (Gg9), JN020643 (Gg10) and JN020644 (Gg11).

Hyper variable regions

Hyper variable (HV) regions were corresponded to nucleotides 47-69 of *Bacillus subtilis* 16S rRNA 5'-GCY TAA YAC ATG CAA GTC GAR CG-3', and 345-365 of *Bacillus subtilis* 16S rRNA, 5'-ACT GCT GCC TCC CGT AGG AGT-3'. HV

regions of *Bacillus* and *Paenibacillus* strains were derived from almost-complete 16S rRNA gene sequences of the strains. Also, HV regions of *Bacillus* and *Paenibacillus* type strains were derived from 16S rRNA bacterial sequences available on the GenBank database. Multiple sequence alignment was performed with the CLUSTAL W program and the calculation of pairwise HV region sequence similarities were achieved using this program (Thompson et al. 1997).

Phylogenetic analysis

16S rRNA and HV genes were aligned using the multiple alignment program, CLUSTAL W program (Thompson et al. 1997). Evolutionary distances were calculated by using Kimura's two parameter model (Kimura 1980). Bootstrap analysis based on 1000 replicates was also conducted in order to obtain confidence levels for the branches (Felsenstein 1985). The phylogenetic trees were constructed using the programs MEGA4 (Tamura et al. 2007).

Experimental infections for bacterial strains

Non-spore forming bacteria and spore-forming bacteria were incubated in a nutrient broth medium at 30°C for 18 h and at 30°C for 72 h (for sporulation), respectively. After incubation, the bacterial density was measured at OD_{600}. And then, all these bacteria were tested against the nymphs of *G. gryllotalpa* (Orthoptera: Gryllotalpidae) at 0.9×10^9, 1.8×10^9, 3.6×10^9 CFU/ml doses within ten days (Ben-Dov et al. 1995; Moar et al. 1995). Five ml of each culture was centrifuged at 3.000 rpm for 10 min. The pellet was re-suspended in 5 ml of sterilized Phosphate Buffer Solution (PBS) and was used in bioassays.

General conditions for experimental infections

Bioassays with nymphs of *G. gryllotalpa* were performed with the bacteria applied on the diet. Diets were prepared from fresh potato and carrot. The diets were placed into individual sterilized glass containers. Bacterial suspensions were applied to the surface of the diets. Ten nymphs collected from fields and reared in laboratory conditions for one week were placed on the diet in containers. Containers were kept at 24±2°C and 60% Relative Humidity on a 12:12 h photo regime, with the diet without bacteria changed after eating. The mortalities of nymphs were recorded every 24 h and all dead nymphs were removed from containers. PBS was used in bioassay as negative control agent.

B. thuringiensis subsp. *kurstaki* (BnBt) was used bioassay as positive control agent. Mortality was recorded 10 days after initiation of the treatment. All bioassays were repeated 3 times on same occasions. At least 30 nymphs were assayed for each isolate in a biotest. Means of actual results of in a biotest were analyzed using one-way analysis of variance (ANOVA) and compared by least significant difference (LSD) test (Minitab 1997).

Results

We found different aerobic strains based on colony morphology among all the bacterial suspensions from the *G. gryllotalpa* insects. Infected insect nymphs present certain symptoms that suggest bacterial infection; they become flaccid, lethargic, and stop eating.

Therefore, total of these strains were examined for morphology, spore formation (Table 1), physiological and biochemical characteristics (Table 2 and 3). Six bacterial strains (Gg1, 3, 5, 6, 7 and 9) were Gram positive and the rest (Gg2, 8, 10, and 11) of them were Gram negative. While the catalase activity was positive for all strains, the oxidase reaction was negative except Gg6. The API 20E (Table 3) and API 50CH systems were also used to characterize the bacterial strains.

Table 1. Morphological characteristics of bacteria from *Gryllotalpa gryllotalpa*

Strain number	Colony color	Colony shape	Colony type	Gram stain	Spore stain	Spore form
Gg1	Cream	Filamentous	Filamentous	+	+	Terminal
Gg2	Cream	Smooth	Round	-	ND	-
Gg3	Cream	Rough	Round	+	+	Central
Gg5	Cream	Rough	Round	+	ND	-
Gg6	Cream	Filamentous	Filamentous	+	+	Subterminal
Gg7	Cream	Rough	Round	+	+	Central
Gg8	Cream	Smooth	Round	-	ND	-
Gg9	White	Rough	Round	+	ND	-
Gg10	Cream	Rough	Round	-	ND	-
Gg11	Cream	Rough	Round	-	ND	-

ND: No determination

Table 2. Biochemical characteristics of bacteria from *Gryllotalpa gryllotalpa*

Strain number	Catalase	Oxidase	Indol	VP	Citrate	Gelatin	Nitrate	Urease
Gg1	+	-	-	-	+	-	+	-
Gg2	+	-	-	-	+	+	+	+
Gg3	+	-	-	-	+	-	+	-
Gg5	+	-	-	-	+	-	-	-
Gg6	+	+	-	-	+	-	+	-
Gg7	+	-	-	-	+	-	-	-
Gg8	+	-	-	-	+	-	+	-
Gg9	+	-	-	-	+	-	-	-
Gg10	+	-	-	+	+	-	+	-
Gg11	+	-	+	+	+	-	+	+

In addition to the results of numerical tests and API test systems, the results from 16S rRNA gene sequences were also used for the characterization of bacterial strains. A total of 1,400 nucleotides of the 16S rRNA from 10 bacterial strains

(Gg1-Gg11) were aligned and compared to sequences of related bacteria in GenBank (Appendix 1). The phylogenetic trees for Gram positive and negative strains were shown at Figure 1 and 2.

Table 3. The results of API 20E panel test system of bacteria from *Gryllotalpa gryllotalpa*

	Gg2	Gg8	Gg9	Gg10	Gg11
Onpg	+	+	+	+	-
Adh	+	+	-	+	-
Lcd	+	+	-	-	-
Odc	+	+	-	+	-
H_2S	-	-	-	-	-
Tda	-	-	-	-	+
Glu	+	+	-	+	+
Man	+	+	-	+	+
Ino	-	-	-	-	+
Sor	+	+	-	+	-
Rha	-	+	-	+	+
Sac	+	+	-	+	-
Mel	+	+	-	+	-
Amy	+	+	-	+	+
Ara	+	+	-	+	-

Moreover, HV sequences of the five Gram positive strains were determined. The HV sequences of these strains were aligned and compared to sequences of related bacteria. Results of the phylogenetic trees were shown at Figure 3 and 4. So, the all of results obtained from characterization works have been confirmed by the phylogenetic trees.

The results of 16S rRNA gene sequences and HV analyses shown that Gg9 does not have any similarity with present species in GenBank. So, it is thought that Gg9 may be a novel species belongs to *Paenibacillus* genus (Figure 4).

Finally, the insecticidal effects of bacterial strains were also determined against the nymphs of *G. gryllotalpa* (Table 4). In each test, the recorded mortality was 100% for Gg1 and Gg5 against the *G. gryllotalpa* nymphs within 10 days at 3.6×10^9 CFU/ml doses. Also, the other results were 25% for Gg9, Gg10 and Gg11, 50% for Gg2 and Gg8, and 75% for Gg3, Gg6 and Gg7 against the *G. gryllotalpa* nymphs within 10 days (ANOVA LSD, $P<0.05$). For the other doses tested, there was not any mortality.

Discussion

There has recently been an increasing interest in finding more effective biological control agent against hazardous insects. Although there are a lot of biological

control studies on *Gryllotalpa gryllotalpa*, to date, there has been no study on the isolation and characterization of bacteria from *G. gryllotalpa* as potential biological control agents.

Table 4. The insecticidal effects of the strains (Gg1-11) on *Gryllotalpa gryllotalpa* nymphs within ten days. ANOVA (F= 52.678; p<0.05. LSD test)

Strains	**Mortality (%±SD)**
Control (PBS)	-
Gg1	100±0
Gg2	50±5
Gg3	75±10
Gg5	100±0
Gg6	75±15
Gg7	75±5
Gg8	50±10
Gg9	25±5
Gg10	25±10
Gg11	25±5

So, we aimed that the isolation and characterization of bacteria isolated from *G. gryllotalpa*, and the determination of the insecticidal potential of these bacterial strains on *G. gryllotalpa* as biological control agents.

According to the results of numerical taxonomy, 16S rRNA and HV analyses, Gg1 may be *Bacillus weihenstephanensis*, *Bacillus thuringiensis* (Bt), and *Bacillus mycoides* at same similarity rates. For determining species of Gg1, firstly it was done PCR by using genomic DNA of Gg1 as template with specific *Cry* primers for *B. thuringiensis* to determine whether Gg1 has *Cry* genes or not. Since there was no band on the agarose gel (Data not shown), it was decided that Gg1 did not include any *Cry* genes. And then, since Gg1, *B. thuringiensis*, and *B. weihenstephanensis* are motile on the contrary *B. mycoides*, it was determined that Gg1 was not *B. mycoides*. So, it was newly researched the 16S rRNA sequence of Gg1 for ^{1003}TCTAGAGATAG motif (Lechner et al. 1998) and found that Gg1 had include the 16S rRNA signature sequence. So, Gg1 was differentiated from *B. thuringiensis* by the presence of ^{998}TCTAGAGATAGA, and from *B. mycoides* by its non-mycoidal colony morphology. So, it was resulted that Gg1 was *Bacillus weihenstephanensis*.

Strain Gg2 has been identified as *Serratia* sp. based on numerical and molecular studies. Since Gg2 had urease enzyme (Table 2), it was identified at species level as *S. ureilytica* that is unique urea-dissolving species in *Serratia* genus (Bhadra et al. 2005).

Strains Gg3, Gg5, Gg6 and Gg7 which belong to *Bacillus* genus according to the results of 16S rRNA (Figure 1) were further analyzed by HV. Based on HV analyses, Gg3 had 99% similarity to *Bacillus simplex* and it was determined as *B. simplex* at species level. According to the results of HV analyses (Figure 3) Gg5

was identified as *B. nanhaiensis*. However, decision was too difficult for Gg6 since three species had same rate of similarity (Appendix 1). Therefore, Gg6 was identified as *Bacillus* sp. at genus level only. Based on 16S rRNA and HV analyses (Figure 1,3), Gg7 which had 98% similarity to *B. gibsoni* was determined as *B. gibsoni* at species level.

As stated above, Gg8 is a member of Enterobacteriaceae family according to the results of the numerical tests and molecular tests. The results shown that there are so many species in terms of the rate of similarity of Gg8 (Appendix 1). In addition, since Gg8 did not use VP such as *Citrobacter* genus, and had same biochemical characteristics (for example, Ldc, Odc, Ino, and Mel) such as *C. braakii*, it was determined as *C. braakii*.

According to results, Gg9 did not resemble to any species on the Table 6, and also, had very low rate of similarity based on 16S rRNA and HV analyses (Figure 2,4), so Gg9 may be a novel species for *Paenibacillus* genus.

Although Gg10 belongs to *Enterobacteriaceae* family, biochemical tests determined that Gg10 did not hydrolyze H_2S, but use ONPG such as *Enterobacter* genus. So, Gg10 was determined as a member of *Enterobacter* sp. according to the results of numerical tests.

For identification of Gg11, initially we used the result of 16S rRNA. However, this result did not prove the identification at species level and then the biochemical results showed that Gg11 shared with same biochemical properties of *Providencia rettgeri*. Thus, Gg11 was determined as *P. rettgeri*.

In conclusion, according to all phenotypic characteristics, API20E and API50CH systems, 16S rRNA gene analyses and HV analyses (Figure 1-4), bacteria were identified as *Bacillus weihenstephanensis* (Gg1), *Serratia ureilytica* (Gg2), *Bacillus simplex* (Gg3), *Bacillus nanhaiensis* (Gg5), *Bacillus* sp. (Gg6), *Bacillus gibsoni* (Gg7), *Citrobacter braakii* (Gg8), *Paenibacillus* sp. (Gg9), *Enterobacter* sp. (Gg10), and *Providencia rettgeri* (Gg11).

Up to now, various bacteria belonging to *Enterobacteriaceae* have been isolated and identified from different insect (Lipa & Wiland 1972; Bucher 1981; Sezen & Demirbag 1999; Sezen et al. 2004; Katı et al. 2007). Determining the symbiotic bacteria in insect species can allow the development of new approaches for biological control (Li et al. 2005). Therefore, many scientists investigated the microbiota of harmful insects (Lipa & Wiland 1972; Fitt & O'Brien 1985; Höfte & Whiteley 1989; Kuzina et al. 2001; Osborn et al. 2002; Sezen et al. 2008b).

Finally, the insecticidal effects of bacterial strains were also determined against the nymphs of *G. gryllotalpa* (Table 4). In each test, the recorded mortality was 100% for Gg1 and Gg5 against the *G. gryllotalpa* nymphs within 10 days. The other results were 25% for Gg9, Gg10 and Gg11, 50% for Gg2 and Gg8, and 75% for Gg3, Gg6 and Gg7 against the *G. gryllotalpa* nymphs within 10 days (ANOVA LSD, $P<0.05$).

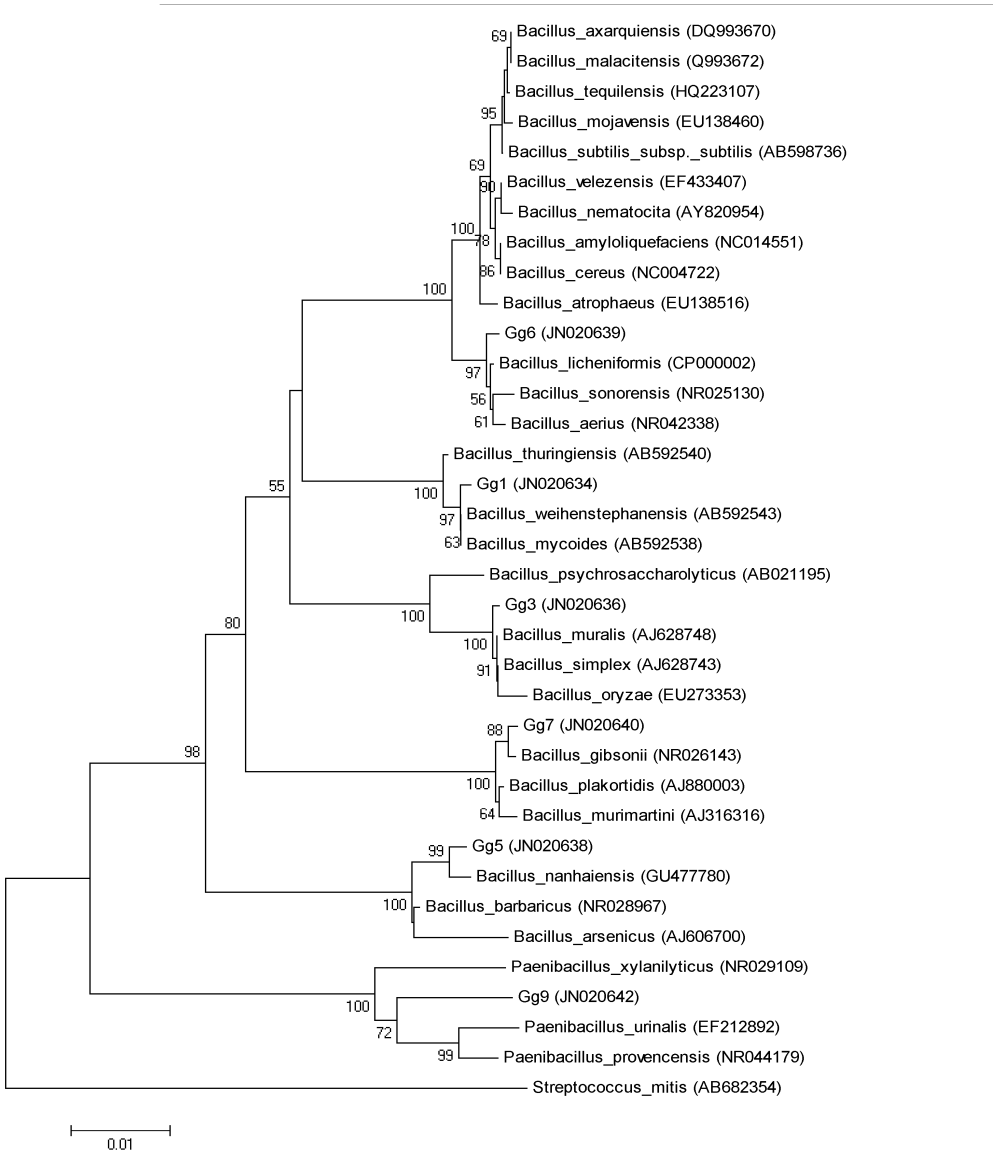

Figure 1. Neighbor-joining tree based on 16S rRNA gene sequences (1186 bases) of bacterial strains. It shows the phylogenetic relationship between Gram positive strains of *G. gryllotalpa* and similarity of Gram positive strains of *G. gryllotalpa*. *Streptococcus mitis* (AB682354) was used as an out-group in the tree. Bootstrap values (expressed as percentages of 1000 replications) >50% are given at nodes. Bar 1 substitution per 100 nucleotides.

Figure 2. Neighbor-joining tree based on 16S rRNA gene sequences (1344 bases) of bacterial strains. It shows the phylogenetic relationship between Gram negative strains of *G. gryllotalpa* and similarity of Gram negative strains of *G. gryllotalpa*. *Thermus rehai* (AF331969) was used as an out-group in the tree. Bootstrap values (expressed as percentages of 1000 replications) >50% are given at nodes. Bar 2 substitutions per 100 nucleotides.

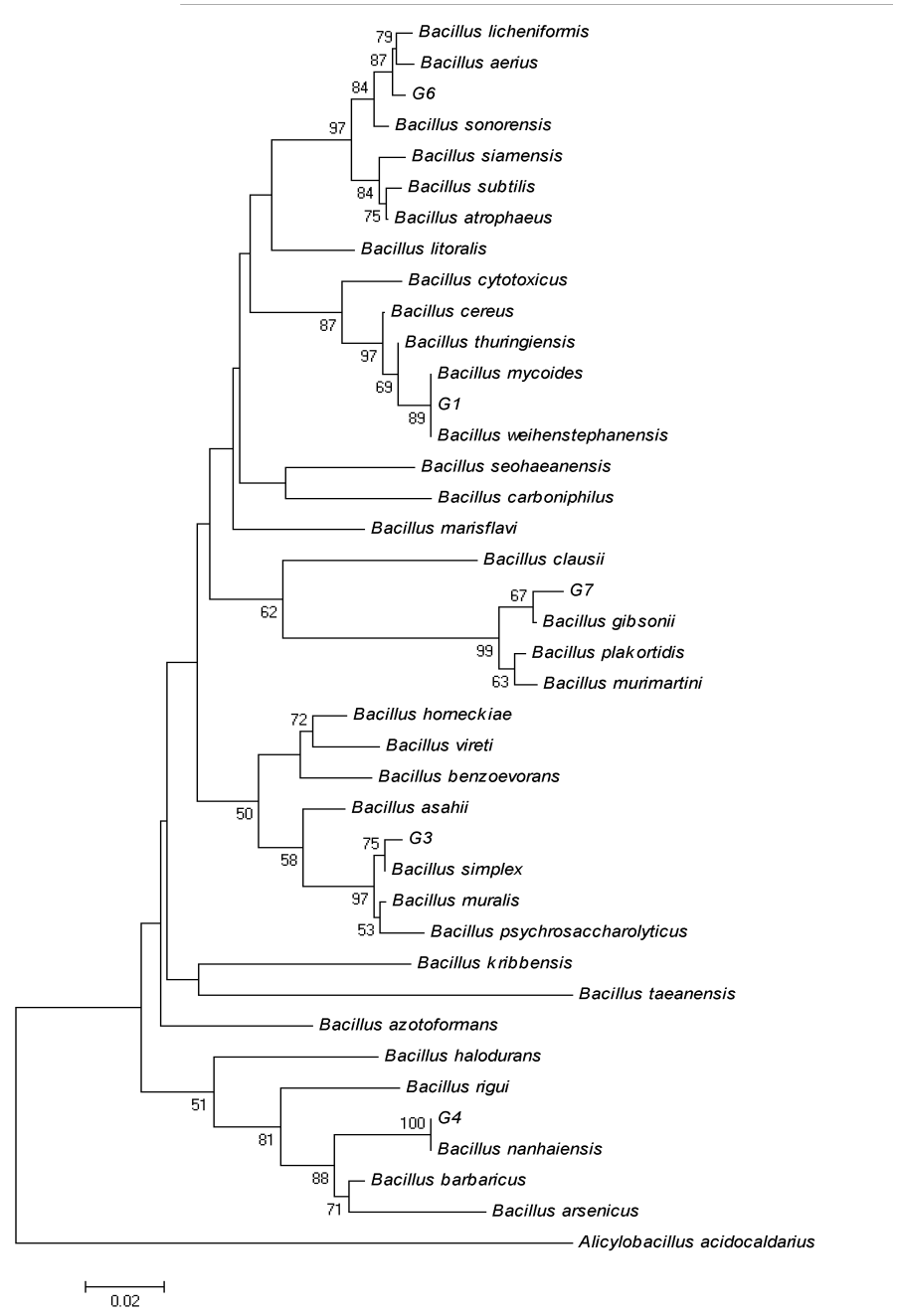

Figure 3. Phylogenetic tree was constructed on the basis of HV sequence data of our five *Bacillus* strains and other related organisms, using the neighbor-joining method. Bar, 2 substitutions per 100 nucleotide positions. *Alicylobacillus acidocaldarius* was used as an out-group in the tree.

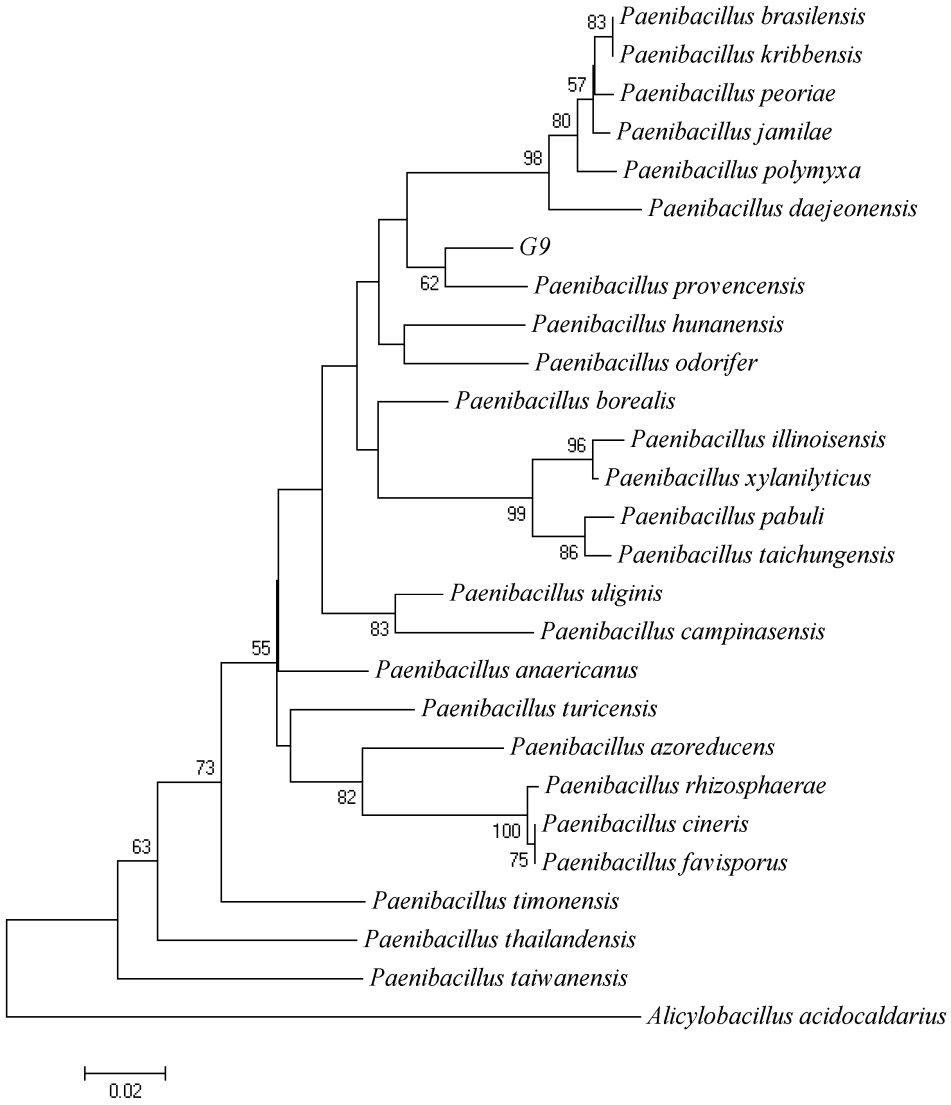

Figure 4. Phylogenetic tree was constructed on the basis of HV sequence data of our one *Paenibacillus* strains and other related organisms, using the neighbor-joining method. Bar, 2 substitutions per 100 nucleotide positions. *Alicylobacillus acidocaldarius* was used as an out-group in the tree.

Since nematodes can be produced on artificial media and cause mortality in the target pest within a few days (Peters 1996), they are considered outstanding candidates for biological control. When the search for natural enemies of mole

crickets began in South America in the early 1980's, it was discovered that 8-50% of crickets collected by calling traps in Uruguay at this time were infected with entomopathogenic nematodes (Parkman & Smart 1996).

A naturally occurring soil fungus, *Beauveria bassiana* (Balsamo) Vuillemin, is an entomopathogenic agent (Feng et al. 1994) of many different species of insects in most orders (Goettel et al. 1990), including Orthoptera-mole crickets. In the *B. bassiana* treatments done by Thompson, Brandenburg & Arends (2003), every treatment had live crickets remaining at the conclusion of the test. But, in this work, all of infected *G. gryllotalpa* nymphs dead duo to Gg1 and Gg5 strains. When two works are compared each other, very clearly, bacterial control is very much efficient than fungus control. According to literature, whatever works are present information about trying with nematodes and fungi were used against the other mole crickets; there is not any information about microbial bioassays on the *G. gryllotalpa*.

However, bacteria-insects interaction is not only pathogenic but also symbiotic. Symbiotic bacteria, ranging from pathogenic to mutualistic and from facultative to obligate, are ubiquitously located in animal guts (Lau et al. 2002). Determining the symbiotic bacteria in insect species can allow the development of new approaches for biological control (Li et al. 2005).

Our results indicate that Gg1 isolated from *G. gryllotalpa* is a strain of *B. weihenstephanensis* which may be valuable potential use for the control of *G. gryllotalpa*. Also, *Bacillus nanhaiensis* (Gg5) has similar potential for the control of *G. gryllotalpa*. Especially, the capacity of Gg1 and Gg5 to control orthopteran nymphs has been a potential biological Bt preparation.

Further studies will involve in providing good sources for developing microbial pesticides against *G. gryllotalpa* and other orthopteran pests.

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