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A new study on the bacteria of *Agrotis segetum* Schiff. (Lepidoptera: Noctuidae) and their insecticidal activities

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Abstract: *Agrotis segetum* Schiff. (Lepidoptera: Noctuidae) is one of the most serious pests of nearly all vegetables in Turkey. In this study, to find a more effective and safe biological control agent, the bacterial flora of *Agrotis segetum* were investigated in larvae collected from different populations in the vegetable fields of the Black Sea region of Turkey, and 9 bacterial isolates were cultured from these populations. Seven of these bacteria were determined and characterized at the species level and the rest were characterized at the genus level. Morphological, physiological, and biochemical characteristics of the bacterial isolates were determined by conventional and routine techniques. Characterization of the isolates was also performed using the VITEK 32 bacterial identification system. In addition, a 16S rRNA gene sequence was determined for these isolates to query against the NCBI genetic database and to construct a phylogenetic tree using closely related species. Consequently, the isolates were identified as *Bacillus cereus* (Ags1), *Bacillus* sp. (Ags2), *Bacillus megaterium* (Ags3), *Enterobacter aerogenes* (Ags4), *Acinetobacter calcoaceticus* (Ags5), *Enterobacter* sp. (Ags6), *Pseudomonas putida* (Ags7), *Enterococcus gallinarum* (Ags8), and *Stenotrophomonas maltophilia* (Ags9). All isolates were determined the first time from the flora of *A. segetum*. Furthermore, the insecticidal activities of the bacterial isolates were tested against *A. segetum* larvae, and *Enterococcus gallinarum* (Ags8) was found to cause the highest insecticidal activity (60%) 8 days after application.

Key words: *Agrotis segetum*, bacterial flora, insecticidal activity

Agrotis segetum Schiff. (Lepidoptera: Noctuidae)'un bakterileri ve bu bakterilerin insektisidal etkileri üzerine yeni bir çalışma

Özet: *Agrotis segetum* Schiff. (Lepidoptera: Noctuidae) Türkiye'de hemen hemen bütün sebzelerin en önemli zararlılarından birisidir. Bu çalışmada, daha etkili ve güvenli biyolojik mücadele etmeni bulmak için *Agrotis segetum*'un bakteriyal florası Karadeniz Bölgesi'ndeki sebze tarlarında farklı populasyonlardan toplanan larvalar üzerinde araştırıldı. Bu populasyonlardan dokuz bakteri izolatı kültüre alındı. Bu bakterilerden yedi tanesi tür seviyesinde, diğerleri ise cins seviyesinde tanımlandı ve karakterize edildi. Bakteriyal isolatların morfolojik, fizyolojik ve biyokimyasal özellikleri, rutin ve geleneksel teknikler kullanılarak belirlendi. İzolatların karakterizasyonu, VITEK 32 bakteriyal tanımlama sistemi kullanılarak da gerçekleştirildi. Buna ilaveten, bütün izolatların NCBI genetik veritabanında karşılaştırmalarını yapmak ve ilişkili türler ile filogenetik yakınlıklarını belirlemek için 16S rRNA gen sekans analizleri yapıldı. Sonuç olarak, izolatlar *Bacillus cereus* (Ags1), *Bacillus* sp. (Ags2), *B. megaterium* (Ags3), *Enterobacter aerogenes* (Ags4), *Acinetobacter calcoaceticus* (Ags5), *Enterobacter* sp. (Ags6), *Pseudomonas putida* (Ags7), *Enterococcus gallinarum* (Ags8) ve *Stenotrophomonas maltophilia* (Ags9) olarak tanımlandı. Bütün izolatlar *A. segetum*'un florasında ilk kez belirlendi. Ayrıca, bakteriyal izolatların insektisidal etkileri *A. segetum* larvalarına karşı test edildi. Uygulamadan 8 gün sonra *Enterococcus gallinarum* (Ags8)'un en yüksek aktiviteye (%60) sebep olduğu bulundu.

Anahtar sözcükler: *Agrotis segetum*, bakteriyal flora, insektisidal etki

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Introduction

Cutworms are important pests for a number of crops in agriculture, horticulture, and forestry around the world (Zethner 1980). The common cutworm (turnip moth), *Agrotis segetum* Schiff. (Lepidoptera: Noctuidae), is a serious pest occurring throughout Europe (including Turkey), Asia, and parts of Africa (Jakubowska et al. 2005). It commonly lives on the ground, where it feeds on seedlings of nearly all vegetable and field crops, most importantly corn, potatoes, beans, peppers, eggplants, okra, lettuce, tobacco, sugar beets, and cabbage, as well as many other plants (The Ministry of Agriculture of Turkey 2008).

Chemical substances such as chlorpyrifos and endosulfan have been utilized to control this pest in Turkey (The Ministry of Agriculture of Turkey 2008). However, recent concerns about the hazardous effects of chemical pesticides on the environment have encouraged scientists to consider finding more effective and safe control agents. Use of some microbial control agents against *A. segetum* was therefore previously studied. Microbial insecticides based on entomopathogenic bacteria, viruses, nematodes, and fungi have been assessed as possible microbial control agents of *A. segetum* (Lipa and Wiland 1972; Ignoffo and Garcia 1979; Lossbroek and Theunissen 1985; Caballero et al. 1991; Steenberg and Ogaard 2000; Yun et al. 2004).

Studies of symbiotic bacteria in insect species allow the development of new approaches for biological control. Symbiotic bacteria are ubiquitously located in animal guts with these symbioses ranging from pathogenic to mutualistic and from facultative to obligate (Lau et al. 2002). In the last few years, several pathogenic bacteria species from insect samples have been developed as pesticides and used successfully in biological control of insects worldwide (Thiery and Frachon 1997). Several studies have been performed to determine the bacterial flora of *A. segetum* (Lipa and Wiland 1972; Turchaninova 1972; Turchaninova 1973), but no such study has been done in Turkey.

Efforts have been increased to discover more effective and safe biological control against hazardous insects to replace potentially hazardous chemical agents (Ince et al. 2008). Moreover, investigating a new pathogen from various environments against a target pest is always desirable. It is also known that the success of control can be higher when native isolates are used against the target insect. Therefore, to find a more effective and safe biocontrol agent against cutworm, we investigated the bacterial flora of *A. segetum* isolated from the Black Sea region of Turkey, utilizing current morphological, biochemical, and molecular techniques. In this study, we isolated 9 bacterial isolates, characterized them in detail, and tested their insecticidal activities against larvae of *A. segetum*.

Materials and methods

Collection of larvae

Larvae of the cutworm, different instars, were used for the isolation of bacteria. Larvae were collected on a daily basis from different tobacco fields in the vicinity of Samsun in May and June of 2007. Larvae were obtained by digging them out of the soil, and they were placed individually into plastic boxes (20 mm) with a little soil and perforated covers to permit airflow. The boxes were transported to the laboratory. Larvae were feed with lettuce at room temperature with a 12:12 photoperiod until the bacterial isolation was done. The lettuce was washed with sterile water and dried before being given to the larvae.

The larvae used in the insecticidal activity tests were provided by Dr. Christer Löfstedt and Erling Jirle (Department of Ecology, Lund University, Sweden) as egg mass.

Isolation and purification of bacterial samples

After macroscopic examination, living larvae that showed general disease symptoms were separated from dead larvae. After that, both dead and living larvae were surface sterilized with 70% alcohol for 2 min and then washed twice with sterile distilled water. The larvae were homogenized in nutrient broth using a glass tissue grinder. The obtained larval extracts were used to prepare a series of dilutions, from 10^{-1} to 10^{-5}. After preparation of the dilutions, 2 methods were used for bacterial purification. In the first, the larval suspension was heated at 80 °C for 10 min in a water bath to eliminate non-spore-forming organisms (Ohba and Aizawa 1986). After that, 100 µL of this suspension was streaked on each nutrient agar (Difco) plate and incubated at 30 °C for 2-3 days. In the

second method, serially diluted suspensions were also spread on nutrient agar (Difco) plates and incubated under the same conditions. At the end of the incubation period, isolates were separated based on the color and morphology of the colonies. Pure cultures of bacterial colonies were then prepared, and these cultures were identified by their morphology, spore formation, and nutritional features; their physiological, biochemical, and molecular characteristics; and by the VITEK 32 bacterial identification system.

Identification of bacterial isolates based on conventional tests

Morphological, physiological, and biochemical properties of the bacterial isolates were determined according to *Bergey's Manual of Systematic Bacteriology*, Volumes 1 and 2 (Palleroni 1984; Kandler and Weiss 1986). Gram staining was performed according to the procedure described by Claus (1992). Endospore staining was performed according to the method of Prescott et al. (1996). Capsule staining was performed by negative staining. The motility of isolates was determined by using a semisolid medium (Soutourina et al. 2001). Physiological tests were performed by using different media and the VITEK 32 bacterial identification system. The pH and NaCl tolerance values were determined in LB broth.

Isolate identification by VITEK 32

The stock culture strains were then subcultured onto tryptic soy agar plates to check their purity. A standard bacterial suspension was prepared in 1.8 mL of 0.45% saline using the VITEK colorimeter for each isolate. The time interval between suspension preparation and card filling was less than 30 min to avoid changes in turbidity. After preparation of the suspension, it was inoculated into the VITEK GNI (for gram-negative bacteria), GPI (for gram-positive bacteria), and CAP (for rod-shaped bacillus bacteria) cards, incubated at 35 °C in the reader incubator module for 18 h, and automatically read hourly by the optical scanner according to the manufacturer recommendations by using bioLiaison software. In addition, since the VITEK system requires the oxidase (gram-negative) and catalase (gram-positive) test results for identification, we also determined features of oxidase and catalase tests (Barry et al. 1982).

Isolate identification by 16S rRNA gene sequencing

Isolation of genomic DNA was performed according to standard phenol/chloroform procedures (Sambrook et al. 1989). DNA pellets were dissolved in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and isolated DNAs were stored at -20 °C until use. PCR amplification of the 16S rRNA gene of bacterial isolates was performed with the following primers: Forward UNI16S-L, 5'-ATTCTAGAGTTTGATCATGGCTCA-3'; and Reverse UNI16S-R, 5'-ATGGTACCGTGTGACGGGCGGTGTGTA-3' (William et al. 1991). PCR conditions were adapted essentially as described by William et al. (1991). Gel-purified PCR products of the 16S rRNA gene fragments from the 9 isolates were cloned directly into the pGEM-T vector cloning system and transformed into *E. coli* JM 101 strain. After that, plasmid isolation was done and plasmid DNA samples were sent to Macrogen for sequencing. The obtained sequences data were submitted to GenBank for accession numbers and were compared with known 16S rRNA gene sequences in the GenBank database using the BLAST search algorithm. Finally, the taxonomic identification of bacterial isolates was determined by comparing the results obtained by conventional tests, VITEK 32, and 16S rRNA gene analysis data.

Insecticidal effects of bacterial isolates

Experimental infections were carried out for the isolates to determine their insecticidal activities. *A. segetum* eggs were obtained from laboratory cultures from Sweden (Department of Ecology, Lund University) and brought to the laboratory. Hatched larvae were grown in the laboratory as described above. Healthy larvae were used for the insecticidal effects of bacterial isolates. Isolates were incubated for 18 h (72 h for *Bacillus* for sporulation) at 30 °C in nutrient broth. After incubation, the density of cells was set at 1.89 at OD_{600}, and 5 mL of culture was centrifuged at 3000 rpm for 10 min (Ben-Dov et al. 1995). The pellet was resuspended in 5 mL of sterilized PBS and used for bioassays. Fresh leaves of lettuce used as food were dipped into the bacterial suspension and placed into individual sterile plastic containers (80 mm in diameter), each containing a single bacterial isolate. For the control group, leaves of lettuces were dipped into sterile water. Ten third instars larvae were placed on the lettuce in containers and kept at 26 ± 2 °C and 60% relative humidity with a 12:12 photoperiod (Mitchell and Smith 1985). The mortalities of larvae were

recorded every 24 h, with all dead larvae removed from the containers. Data were evaluated by using Abbott's formula (Abbott 1925).

Phylogenetic analysis of bacterial isolates

The evolutionary relationship of the 9 bacterial isolates and 28 closely related species were evaluated. An approximately 1400 bp segment of 16S rRNA was used for phylogenetic analysis. Sequences were assembled and edited with BioEdit and aligned (Hall 1999). Cluster analyses of the sequences were performed using BioEdit (version 7.09) with ClustalW. To evaluate the phylogenetic relationship of isolates, neighbor-joining analysis was performed on aligned sequences by using the Kimura 2-parameter test with MEGA 4.0 software (Tamura et al. 2007). Alignment gaps were treated as missing data. The reliability of the phylograms was tested by bootstrap analysis with 1000 replicates using MEGA 4.0.

Results

A total of 9 isolates were finally selected and characterized according to morphology, spore formation, nutritional features, physiological and biochemical characteristics, results of the VITEK 32 system, and analysis of the 16S rRNA gene sequence (Tables 1, 2, 3, 4, and 5).

On agar plates, 8 isolates were cream-colored and 1 isolate was orange (Table 1). Colonies of all isolates were smooth, except for Ags1, which was filamentous after 1 day of incubation on a nutrient agar plate at 30 °C. Two spore-forming bacteria (Ags1 and Ags3) and 7 non-spore-forming bacteria were identified. Seven isolates were rod-shaped and 2 isolates were coccobacillus. Four isolates (Ags1, Ags2, Ags3, and Ags8) were gram-positive and the rest of the isolates were gram-negative. Motility was observed in 6 isolates (Ags1, Ags4, Ags5, Ags6, Ags7, and Ags8). All

Table 1. The morphological characteristics of the bacterial isolates.

Isolate number	Ags1	Ags2	Ags3	Ags4	Ags5	Ags6	Ags7	Ags8	Ags9
Color of colonies	Cream	Orange	Cream	Cream	Cream	Cream	Cream	Cream	Cream
Shape of colonies	Irregular	Smooth	Smooth	Smooth	Smooth	Smooth	Round	Smooth	Smooth
Gram stain	+	+	+	-	-	-	-	+	-
Shape of bacteria	Bacillus	Bacillus	Bacillus	Bacillus	Coccobacillus	Coccobacillus	Bacillus	Bacillus	Bacillus
Spore stain	+	-	+	-	-	-	-	-	-
Spore shape	ND[b]	ND	ND	ND	ND	ND	ND	ND	ND
Spore form	Central	-	Central	-	-	-	-	-	-
Capsule	+	+	+	+	+	+	+	+	+
Turbidity when grown in NB[a]	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid
Motility	+	-	-	+	+	+	+	+	-

[a]NB: Nutrient Broth; [b]ND: No Data

Table 2. The physiological characteristics of the bacterial isolates.

Isolate number	Ags1	Ags2	Ags3	Ags4	Ags5	Ags6	Ags7	Ags8	Ags9
Growth in 2% NaCl	+	+	+	+	+	+	+	-	+
Growth in 5% NaCl	-	-	+	+	-	+	+	-	-
Growth in 7% NaCl	-	-	+	+	-	+	-	-	-
Growth in 12% NaCl	-	-	-	-	-	-	-	-	-
Growth in pH 3	-	-	-	-	-	-	-	-	-
Growth in pH 4	-	-	-	+	+	+	+	-	-
Growth in pH 5	+	-	+	+	+	+	+	+	+
Growth in pH 5.5	+	-	+	+	+	+	+	+	+
Growth in pH 6	+	+	+	+	+	+	+	+	+
Growth in pH 7	+	+	+	+	+	+	+	+	+
Growth in pH 8	+	+	+	+	+	+	+	+	+
Growth in pH 9	+	+	+	+	+	+	+	+	+
Growth in pH 10	-	-	-	+	-	-	-	+	+
Growth in pH 12	-	-	-	-	-	-	-	-	-

Table 3. The biochemical characteristics of the bacterial isolates.

Isolate number	**Ags1**	**Ags2**	**Ags3**	**Ags4**	**Ags5**	**Ags6**	**Ags7**	**Ags8**	**Ags9**
Nitrate reduction	+	+	+	+	+	+	+	+	+
KIA	Basic	Basic	Basic	Basic	Basic	Basic	Basic	Acid	Basic
Oxidase	+	-	-	+	+	-	+	-	+
Methyl red test	+	ND[a]	+	-	+	+	-	+	-
Voges Proskauer	-	ND	-	-	-	-	-	-	-
Citrate	-	-	-	+	+	-	+	-	-
Hydrolysis of urea	+	+	+	+	+	+	+	+	+
Indol	-	-	-	-	-	+	-	ND	-
Gelatin hydrolysis	+	-	-	-	-	-	-	-	+
Catalase	+	+	+	+	+	+	+	-	+
Coagulase			-						
Hydrolysis of starch	+	+	+	+	+	-	-	-	-
Sucrose	+	-	+	+	-	-	-	+	-
Esculin	+	-	+	+	-	+	-	+	+
Lactose (10%)	ND	-	ND	+	+	+	-	+	+
Trehalose	+	ND	+	ND	ND	ND	ND	+	ND
Arginine	ND	-	ND	-	-	-	+	+	-
Mannitol	-	-	+	+	-	+	-	+	-
Arabinose	-	-	+	+	+	+	+	+	-
Urea	ND	-	ND	-	-	-	-	-	-
Raffinose	-	-	+	+	-	-	-	+	-
Ribose	-	ND	-	ND	ND	ND	ND	-	ND
Xylose	-	-	-	-	+	-	+	+	+
Sorbitol	-	-	+	+	-	-	-	-	-
DP 300	ND	-	ND	-	-	-	+	ND	-
Maltose	+	-	+	+	-	+	-	ND	+
Inositol	-	-	-	-	-	-	-	ND	-
TLA (10% lactose)	ND	-	ND	+	+		-	ND	-
Glucose (oxidative)	ND	-	ND	+	+	+	+	ND	-
Adonitol	ND	-	ND	+	-	-	-	ND	-
Glucose	+	-	+	+	-	+	-	ND	-
Growth control	ND	+	ND	+	+	+	+	ND	-
Malonate	ND	-	ND	+	+	+	+	ND	+
Coumaric acid	ND	-	ND	-	+	-	+	ND	-
Acetamide	ND	-	ND	-	-	-	+	ND	-
Plant indican	ND	-	ND	-	-	+	-	ND	+
Rhamnose	ND	-	ND	+	-	+	-	ND	-
The mark of oxidase	ND	-	ND	+	+	-	+	ND	+

[a]ND: No Data
Note: The 1st through 28th tests were conventional; the rest were performed with VITEK 32.

Table 4. Results of identification of isolates according to VITEK 32 and 16S rRNA.

Isolate Number	**VITEK 32 System**	**16S Gene Sequence**	**16S Gene Similarity (%)**	**Accession Numbers**
Ags1	91% *Bacillus cereus*	*Bacillus thuringiensis* serovar *kurstaki* BnBt	99%	EU153549
		Bacillus weihenstephanensis 32	99%	AB334765
		Bacillus cereus strain AH 521	99%	AF290554
		Bacillus mycoides CIP 103472	99%	AM747229
		Bacillus thuringiensis 2000031482	99%	AY138290
Ags2	99% *Acinetobacter lwoffii/junii*	*Bacillus arsenicus* strain S8-06	99%	EU620414
		Bacillus sp. JL872	99%	DQ985053
		Bacillus barbaricus VII-B3-A2	98%	AJ422145
		Bacillus gelatini 506	94%	DQ350818
Ags3	99% *Bacillus megaterium*	*Bacillus megaterium* P2P5	99%	EU221388
		Bacillus subtilis C14-1	99%	EU257452
		Bacillus flexus L2S2	99%	EU221413
Ags4	Unidentified Organism	*Enterobacter asburiae* J2S4	99%	EU221358
		Enterobacter aerogenes WAB1906	98%	AM184247
		Enterobacter sp. 3-1t	99%	EU543690
		Pantoea agglomerans WAB1872	99%	AM184214
		Citrobacter freundii strain GM1	99%	DQ133536
		Enterobacter cloacae 766	98%	AM778415
		Erwinia aphidicola GTC 1688	99%	AB273744
Ags5	99% *Acinetobacter calcoaceticus-baumannii*	*Acinetobacter calcoaceticus* HPC253	99%	AY346313
		Acinetobacter rhizosphaerae PhyCEm-460	99%	AM921638
		Acinetobacter baumannii Ab8	99%	AY847284
Ags6	34% *Enterobacter cancerogenus*	*Pantoea agglomerans* HDDMN03	99%	EU879089
		Enterobacter sp. TUT1014	98%	AB098582
		Citrobacter sp. BinzhouCLT	98%	AF530068
		Enterobacter aerogenes zjs04	98%	DQ857896
Ags7	96% *Pseudomonas flourescens/putida*	*Pseudomonas plecoglossicida* FPC951	99%	AB009457
		*Pseudomonas putida*ATCC 17390	99%	AF094737
		Pseudomonas taiwanensis BCRC 17751	99%	EU103629
Ags8	91% *Entorococcus gallinarum*	*Enterococcus gallinarum* LMG 13129	99%	AJ301833
		Enterococcus casseliflavus strain F32	99%	EU151766
		Enterococcus flavescens CECT4481T	99%	AJ420802
		Enterococcus saccharolyticus	99%	ESU30931
Ags9	70% *Stenotrophomonas maltophilia*	*Xanthomonas retroflexus* BBTR24	99%	DQ337602
		Stenotrophomonas maltophilia S-1	99%	AB194708

Table 5. Taxonomic identification of bacterial isolates of *A. segetum*.

Isolate Number	Suggested identification	Accession numbers of 16S gene
Ags1	*Bacillus cereus*	FJ380120
Ags2	*Bacillus* sp.	FJ380121
Ags3	*Bacillus megaterium*	FJ380122
Ags4	*Enterobacter aerogenes*	FJ380123
Ags5	*Acinetobacter calcoaceticus*	FJ380124
Ags6	*Enterobacter* sp.	FJ380125
Ags7	*Pseudomonas putida*	FJ380126
Ags8	*Enterococcus gallinarum*	FJ380127
Ags9	*Stenotrophomonas maltophilia*	FJ380128

isolates caused turbidity in the nutrient broth medium.

The physiological and biochemical characteristics of the isolates are reported in Tables 2 and 3.

For a more detailed identification of these bacterial isolates, the VITEK 32 identification system was used and a 16S rRNA gene sequence analysis was performed (Table 4). According to the VITEK 32 identification system, among the 9 isolates tested, 8 isolates were correctly identified at the species level. Only one isolate could not be identified.

After analyzing the conclusions of the conventional tests, the VITEK 32 identification system, and the 16S rRNA gene sequences, we were able to identfy 7 isolates at the species level [Ags1 (*Bacillus cereus*), Ags3 (*Bacillus megaterium*), Ags4 (*Enterobacter aerogenes*), Ags5 (*Acinetobacter calcoaceticus*), Ags7 (*Pseudomonas putida*), Ags8 (*Enterococcus gallinarum*), and Ags9 (*Stenotrophomonas maltophilia*)] and were able to identify 2 isolates at the genus level [Ags2 (*Bacillus* sp.) and Ags6 (*Enterobacter* sp.)] (Table 5). Figure 1 shows the phylogenetic analysis of the bacterial isolates and closely related species based on the 16S rRNA gene sequencing.

In addition, we tested the insecticidal activity of the bacterial isolates against third instar *A. segetum* larvae. Isolate Ags8 showed the highest insecticidal activity with 60% mortality 8 days after application (Figure 2). Isolates Ags1, Ags2, Ags3, Ags4, Ags5, Ags6, Ags7, and Ags9 had 30%, 10%, 40%, 10%, 20%, 30%, 20%, and 30% mortality 8 days after application, respectively (Figure 2).

Discussion

In this study, we characterized 9 bacterial isolates based on morphological, nutritional, and physiological characteristics; biochemical studies; and the VITEK 32 system and 16S rRNA sequences. Based on these tests, the bacterial flora of *Agrotis segetum* consisted of *Bacillus cereus* (Ags1), *Bacillus* sp. (Ags2), *B. megaterium* (Ags3), *Enterobacter aerogenes* (Ags4), *Acinetobacter calcoaceticus* (Ags5), *Enterobacter* sp. (Ags6), *Pseudomonas putida* (Ags7), *Enterococcus gallinarum* (Ags8), and *Stenotrophomonas maltophilia* (Ags9) (Table 5). For bacterial identification, conventional results are sufficient for identifying a new bacterial isolate most of the time, but occasionally it is very difficult to identify some bacteria based only on conventional tests (Bahar and Demirbag 2007). Hence, we supported identifications of bacterial isolates by using the VITEK 32 bacterial identification system and 16S rRNA gene sequence analysis. Some strains require further study for detailed characterization.

Although Lipa and Wiland (1972) determined 8 bacterial isolates from *A. segetum* larvae, including *Aerobacter cloacae*, *Aerobacter* sp., *Bacillus thrungiensis*, *Brevibacterium maris*, *Escherichia coli*, *Escherichia freundii*, *Micrococcus luteus*, and *Pseudomonas flourescens*, all of the isolates in our study were determined for the first time from the bacterial flora of *A. segetum*. Three species were determined to belong to the genus *Bacillus*. These isolates were identified as *Bacillus cereus* (Ags1), *Bacillus* sp. (Ags2), and *B. megaterium* (Ags3). The levels of 16S rRNA sequence similarity among the isolates are shown in Table 4. The differences between isolates of previous studies and this study are probably due to insufficient identification methods utilized in previous studies. Unfortunately, it is not possible to compare these isolates because there is no culture collection from the previous studies.

A common soil organism, *Bacillus cereus*, has been found to be a pathogen for insects on several occasions, and this species has been isolated from

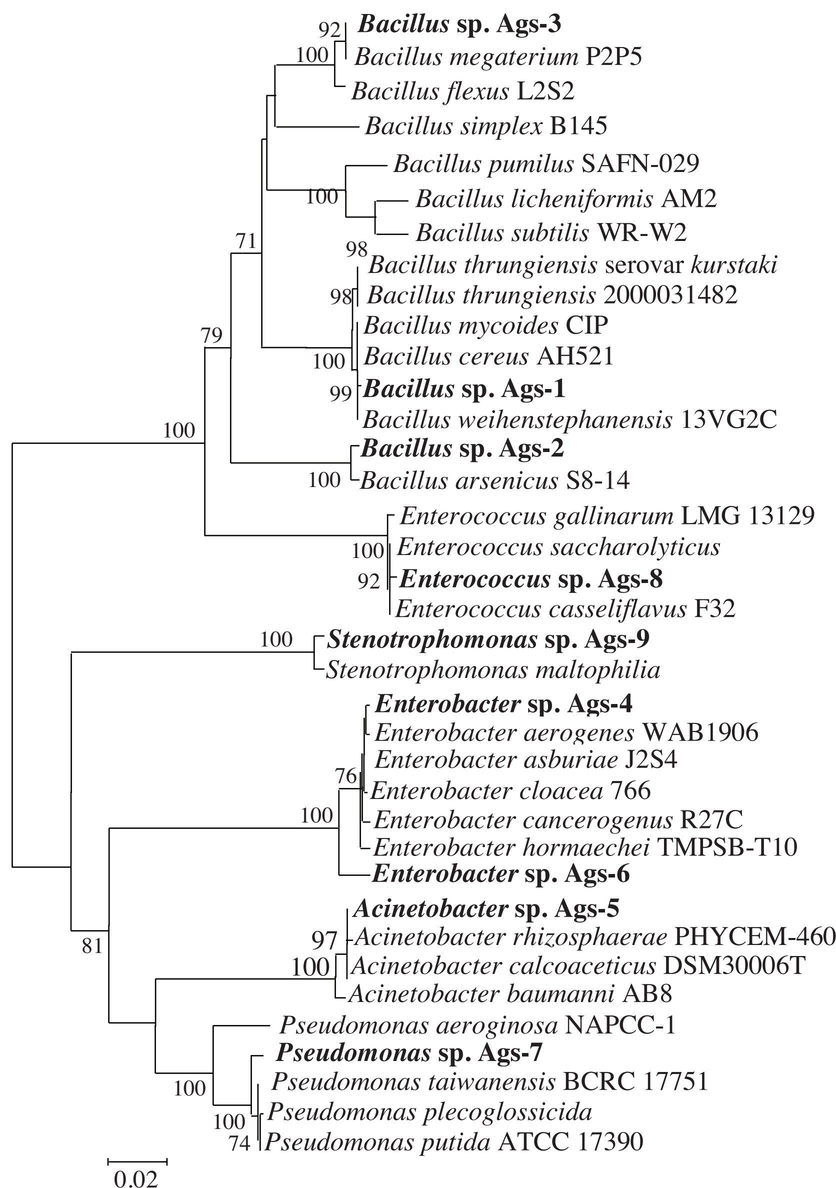

Figure 1. Phylogenetic analysis of bacterial isolates from *A. segetum* and closely related species based on 16S rRNA gene sequencing. Bootstrap values are shown next to nodes and are based upon 1000 replicates using MEGA 4.0.

several insect species (Kuzina et al. 2001; Sezen et al. 2005). Like *B. cereus*, *B. megaterium* is common in insect populations (Osborn et al. 2002). So far, several *Bacillus* species have been isolated from different insect species and are very common in insect populations (Demir et al. 2002; Murrell et al. 2003; Yilmaz et al. 2006; Ince et al. 2008).

Like the genus *Bacillus*, several species of *Enterobacter* have been isolated from several insect species and used for biological control purposes (Sandra and Douglas 2004; Bahar and Demirbag 2007). Other isolates determined in this study [*Acinetobacter calcoaceticus* (Ags5), *Pseudomonas putida* (Ags7), *Enterococcus gallinarum* (Ags8), and *Stenotrophomonas maltophilia* (Ags9)] have also already been shown to be common in insect populations (Yilmaz et al. 2006; Bahar and Demirbag 2007; Cox and Gilmore 2007).

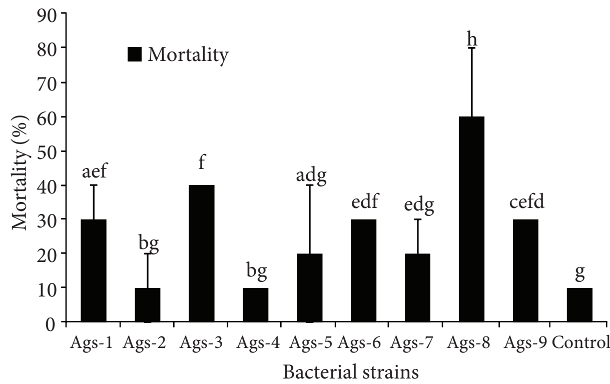

Figure 2. Mortality of the third instar larvae of *Agrotis segetum* 8 days after application of bacterial isolates. Mortality data were corrected according to Abbott's formula. Different letters show the difference between isolates according to the LSD multiple comparison test ($P < 0.05$). Bars show standard deviation. Ags1: *Bacillus cereus*, Ags2: *Bacillus* sp., Ags3: *Bacillus megaterium*, Ags4: *Enterobacter aerogenes*, Ags5: *Acinetobacter calcoaceticus*, Ags6: *Enterobacter* sp., Ags7: *Pseudomonas putida*, Ags8: *Enterococcus gallinarum*, Ags9: *Stenotrophomonas maltophilia*.

In all bioassays, the highest insecticidal infectivity determined in the *A. segetum* larvae was 60% for *Enterococcus gallinarum* (Ags8). The other isolates, Ags1, Ags2, Ags3, Ags4, Ags5, Ags6, Ags7, and Ags9, had 30%, 10%, 40%, 10%, 20%, 30%, 20%, and 30% mortality, respectively (Figure 2). The results of the bioassays indicated that all isolates (spore-forming and non-spore-forming) are pathogenic to the pest at different ratios, except for Ags2 and Ags4 (Figure 2). Lipa and Wiland (1972) observed the highest insecticidal activity on isolate *Pseudomonas fluorescens* 94-83a with 88% mortality by injection of bacterial suspension. The same strain showed 17% mortality in *A. segetum* larvae by microfeeding. However, we determined the highest insecticidal activity in isolate Ags8 (*Enterococcus gallinarum*) 8 days after application by direct feeding of larvae with lettuce. That means that this isolate can be more pathogenic to *A. segetum* larvae under field conditions.

Enterococci are gram-positive and naturally occur in large numbers in the intestines of mammals, birds, reptiles, and insects (Lukasova and Sustackova 2003). These bacteria are not regarded as primary pathogens, but they are generally recognized as nosocomial pathogens worldwide (Linden and Miller 1999). Some studies have shown the isolation of *Enterococcus gallianarum* from several insect species (Xiang et al. 2006; Cox and Gilmore 2007). Although many Enterococci species are known as common symbionts in the gastrointestinal tracts of domestic animals, with this study, we showed for the first time that *Enterococcus gallinarum* (Ags8) has an important mortality value against *A. segetum*.

In conclusion, we determined members of the bacterial flora of *Agrotis segetum* larvae. Compared to previous studies of the bacterial flora of *A. segetum*, all bacterial isolates determined in this study were different. Isolate Ags8 appears to be a promising biocontrol agent for use against this pest. Further research will investigate the potential of these bacteria as a biocontrol agent against *A. segetum*.

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