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Determination and pathogenicity of the bacterial flora associated with the spruce bark beetle, *Ips typographus* (L.) (Coleoptera: Curculionidae: Scolytinae)

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Abstract: The Eurasian spruce bark beetle, *Ips typographus* (L.) (Coleoptera: Curculionidae: Scolytinae), is one of the most serious pests of spruce trees. We identified 8 bacterial isolates from this pest using conventional bacteriological tests (API 20E and API 50CH strips, and VITEK system (bioMerieux) analysis) and 16S rRNA gene sequence analysis. Based on these studies, all isolates could be identified to the genus or species level as *Bacillus sphaericus* (It1), *Acinetobacter* sp. (It2), *Kluyvera cryocrescens* (It3), *Acinetobacter* sp. (It4), *Vagococcus* sp. (It5), *Acinetobacter* sp. (It6), *Proteus vulgaris* (It7), and *Serratia liquefaciens* (It8). We also evaluated the pathogenicity of these bacteria on adults of *I. typographus*. The insecticidal activity of the bacterial isolates at a concentration of 1.8×10^9 bacteria/mL, within 10 days, was 13.3% for *B. sphaericus* (It1), 16.6% for *Acinetobacter* sp. (It2 and It4), 23.3% for *P. vulgaris* (It7), and 53.3% for *S. liquefaciens* (It8). Since only It8 produced significantly increased mortality relative to the control, the bacterium *S. liquefaciens* may have potential as a biological control agent against the Eurasian spruce bark beetle.

Key words: Bacterial identification, insecticidal activity, *Ips typographus*, microbial control

Sekiz dişli kabuk böceği *Ips typographus* (L.) (Coleoptera: Curculionidae: Scolytinae)'un bakteriyal florasının belirlenmesi ve patojenitesi

Özet: Sekiz dişli kabuk böceği, *Ips typographus* (L.) (Coleoptera: Curculionidae: Scolytinae), en önemli ladin zararlılarından biridir. Bu zararlıdan konvensiyonel bakteriyolojik testler (API 20E ve API 50CH kitleri ve VITEK sistem bioMerieux) ve 16S rRNA gen sekans analizleri kullanılarak sekiz bakteriyal izolat belirlendi. Yapılan çalışmalara dayanılarak tüm izolatlar cins ya da tür seviyesinde tanımlandı; *Bacillus sphaericus* (It1), *Acinetobacter* sp. (It2), *Kluyvera cryocrescens* (It3), *Acinetobacter* sp. (It4), *Vagococcus* sp. (It5), *Acinetobacter* sp. (It6), *Proteus vulgaris* (It7) ve *Serratia liquefaciens* (It8). Ayrıca bu bakteriyal izolatların *I. typographus* erginleri üzerinde bioassayleri de yapıldı. Bakteriyal izolatların 10 gün ve $1{,}8 \times 10^9$ bakteri/mL konsantrasyondaki insektisidal aktivite sonuçları: *B. sphaericus* (It1) için % 13,3, *Acinetobacter* sp. (It2 and It4) için % 16,6, *P. vulgaris* (It7) için % 23,3 ve *S. liquefaciens* (It8) için ise % 53,3'dir. Sadece kontrole göre en yüksek insektisidal aktiviteyi It8 ürettiği için, *S. liquefaciens* sekiz dişli kabuk böceğine karşı biyolojik kontrol potansiyeline sahip olabilir.

Anahtar sözcükler: Bakteriyal tanımlama, insektisidal aktivite, *Ips typographus*, mikrobiyal kontrol

Introduction

Some bark beetle species are able to attack and kill living trees and may thereby cause large economic losses. The eight-toothed spruce bark beetle, *Ips typographus* (L.) (Coleoptera: Curculionidae: Scolytinae), is one of the most serious pests of mature spruce, *Picea abies* (L.) H.Karst., in Turkey and Eurasia (1,2). This beetle, as with many other so-called aggressive bark beetles, introduces phytopathogenic fungi that help exhaust the tree's defenses against attack (3). The beetles must attack a standing tree in large numbers to ensure that enough fungi are introduced to kill the tree before it can mobilize its defenses and repel or kill the beetles.

Many methods have been attempted to control *I. typographus*. The most effective measures are to prevent population build-up of the beetles by maintaining healthy, vigorous stands, and to remove infested trees from the forest before the new generation of adult beetles emerges. Forest management is recommended in order to increase the stability and vitality of forest stands (1,4,5). Mass trapping with pheromone-baited traps or trap trees has also been used to suppress beetle populations and prevent outbreak conditions (6,7). Mechanical and chemical control measures have also been utilized for a long time, such as pheromone traps, including components (*S*)-*cis*-verbenol and methyl butanol, and tree traps. However, these conventional control methods are both expensive and hard to apply, due to topographic obstacles. They also have detrimental effects on predators and parasites of the beetle. Thus, a different method should be employed in controlling this beetle.

Insect predators, parasitoids, and parasites, such as viruses (8), rickettsia, nematodes, fungi (9), and bacteria (10), have also been identified and used for biological control in some associated insects. The major species of bacteria used in insect control are spore-forming *Bacilli*, with the best-known example being *Bacillus thuringiensis*, which has been used primarily for the control of Diptera and Lepidoptera (11). More recently, convincing evidence that the digestive tracts of terrestrial arthropods are niches not only of *B. cereus*, but also of *B. thuringiensis* and *B. mycoides*, has been provided by Swiecicka and Mahillon (12). Studies of symbiotic bacteria in insect species are also allowing the development of new approaches for biological control. Symbiotic bacteria are ubiquitously located in animal guts and are involved in symbioses that range from pathogenic to mutualistic and from facultative to obligate (13).

Bacterial symbionts are thought to enable their hosts to survive on restrictive diets by providing nutritional supplements such as amino acids and vitamins (14-16). 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 (17,18). The Chagas disease vector, *Rhodnius prolixus*, harbors the symbiotic bacteria *Rhodococcus rhodnii*. Beard et al. (17,18) 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 (19). The determination of the bacterial flora of important pest insects is an important first step in the process of using bacteria in biological control.

In this study, we described the isolation and further characterization of bacteria from the adults of *I. typographus*. Using conventional tests and sequence analysis of the bacterial 16S rRNA gene, we identified 8 bacterial isolates to at least the genus level. In order to find and identify a new toxic bacterial isolate against the adults of *I. typographus*, we isolated the entomopathogenic bacteria from the adults of *I. typographus*, characterized them in detail, and tested for their insecticidal activity.

Materials and methods

Collection of insects

Adults of *I. typographus* were collected from spruce forests in different areas in Artvin, Turkey, in June 2003. Collected insects were brought to the Microbiology Laboratory of the Biology Department of Karadeniz Technical University in Trabzon, Turkey.

Isolation of bacteria from *I. typographus*

Adult insects were examined macroscopically and distinguished as dead or healthy. We used the dead adult insects to search for the entomopathogenic bacteria and the healthy adult insects to determinate the bacterial flora. They were then surface-sterilized in 70% alcohol to remove possible contaminants (20,21). The adults were separated into groups as dead or healthy, including 5 adults at a time, and they were homogenized in a nutrient broth medium with a glass tissue grinder. This process was repeated 3 times for the healthy and dead specimens, separately and on different occasions. In total, 30 adult insects were used in the isolation step. The suspension was filtered twice through 2 layers of cheesecloth to remove debris (21). It was then diluted to 10^{-8} (22), and 100 µL of each suspension of dead and healthy adults was plated on nutrient agar. Plates were incubated at 30 °C for 24-48 h. According to the color and morphology of the colonies, the bacteria were separated on the culture media. Colonies were restreaked on nutrient agar petri plates until a pure culture was obtained. Pure cultures of isolates were coded as abbrevations of insect names and isolation numbers. While It1, It2, It3, It4, and It5 were isolated from healthy adults of *I. typographus*, It6, It7, and It8 were isolated from dead adults of *I. typographus*, and pure cultures were stored at -80 °C in 20% glycerol.

Biochemical characterization of bacterial isolates

Bacterial cultures were identified by various tests, such as utilization of organic compounds, spore formation, NaCl tolerance, optimum temperature, motility test, catalase test, and oxidase test. Three sets of nutrient broth were prepared containing 4%, 6.5%, and 8.5% NaCl, respectively. The growth of isolate at different salt concentrations was tested using nutrient broth as organic substrate and a control broth without any NaCl supplementation. Semisolid motility test medium may also be used to detect motility. The agar concentration (0.3%) was sufficient to form a soft gel without hindering motility. A tetrazolium salt (TTC) was incorporated into the medium. When motile organisms are stabbed into soft agar, they swim away from the stab line. Bacterial metabolism reduces the TTC-producing formazan, which is red in color. Catalase activity was determined by the production of bubbles from 3% (v/v) H_2O_2, and oxidase activity was determined using 1% (w/v) N,N,N',N'-tetramethyl-p-phenylenediamine. Biochemical features of the bacterial isolates were determined using the VITEK system (bioMerieux) and API 20E and API 50CH (bioMerieux) strips. API and VITEK are automated identification systems for bacteria that have been developed and commercialized. Analysis of the results was based on the computerized software of VITEK, according to the percent identification accuracy (ID%), an estimate of how closely the profile corresponds to the taxon relative to all other taxa in the database, and an estimate of how closely the profile corresponds to the most typical set of reactions for the stated taxon. However, API systems were used only to characterize the bacterial isolate, because, as pointed out by Behrendt et al. (23) and Peix et al. (24), the identification of nonclinical isolates is often wrong with these systems.

DNA templates and PCR analysis

For 16S rRNA gene sequencing, DNA was extracted from the bacteria as described previously (25). PCR amplification of 16S rRNA genes was performed with oligonucleotide primers. Reactions were routinely carried out in quantities of 50 µL; 1 µL of template DNA was mixed with reaction buffer, 150 µM (each) deoxynucleoside triphosphate, 0.5 µM (each) primers, and 0.5 U Taq DNA polymerase. A 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 a thermal cycler (Bio-Rad). Amplification was performed with a 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 1.2% agarose gel electrophoresis. The gel was then examined with the BioDoc Analyze System (Biometra GmbH, Göttingen, Germany). These fragments were cloned with the pGEM-T Easy Vector (Promega) and transformed to the *Escherichia coli* JM101 strain. Sequencing of the 16S rRNA genes was performed by Macrogen, Inc. (Seoul, Republic of Korea). The resulting 16S rDNA sequences were manually aligned,

and the sequences obtained were compared with those from GenBank using the BLAST program (26). After comparison, species that shared a similarity of between 97%-100% were recorded for further identification.

Preparation of bacterial isolates for bioassay

Bacteria were incubated in a nutrient broth (NB) medium containing beef extract, yeast extract, peptone, and sodium chloride at 30 °C for 18 h. After incubation, the density of each bacterial culture was adjusted to 1.89 at OD_{600} (1.8×10^9 cfu) (27,28), and 3 mL of this culture was centrifuged at 3000 rpm for 10 min. The pellet was resuspended in 0.75 mL of sterilized PBS, and 0.25 mL of bacterial suspension was used in bioassays.

General conditions for experimental infections

Bioassays were performed with adults of *I. typographus*. A diet was prepared for the specimens from the bark of *Picea orientalis*. The bark was cut into rectangles (2 × 3 cm), and one piece of bark was placed into each individual sterilized petri dish (80 mm in diameter) for the adults. Bacterial isolates prepared in phosphate buffered saline (PBS) were both injected into the bark and applied to the surface. The control group was fed with spruce bark sprayed with PBS. Ten adults were placed on each petri dish. Plates were kept at 26 ± 2 °C and 60% relative humidity on a 12:12 h photoperiod (29). During this time, the adults tried to construct galleries in the small bark pieces. Mortality of adults was recorded after 10 days. Infectivity tests were also carried out with the untreated controls, which had only PBS. At least 30 adults were assayed for each isolate. All bioassays were repeated 3 times on different occasions. Mean mortality by isolate was analyzed using one-way analysis of variance (ANOVA), and isolates were compared using the least significant difference (LSD) test (30).

Results

The 8 candidate bacteria from the bark beetle homogenate were efficiently selected. Based on all tests, we were able to identify all isolated bacteria, to at least the genus level, as *Bacillus sphaericus* (It1), *Acinetobacter* sp. (It2), *Kluyvera cryocrescens* (It3), *Acinetobacter* sp. (It4), *Vagococcus* sp. (It5), *Acinetobacter* sp. (It6), *Proteus vulgaris* (It7), and *Serratia liquefaciens* (It8). Bacterial isolates from It1 to It5 were isolated from live adults, and those from It6 to It8 were isolated from dead adults.

Isolate 1 (It1): This gram-positive rod and non-spore-forming bacterium was identified as *Bacillus sphaericus*. The other properties of It1 are shown in Tables 1-3. The VITEK system (BACIL) showed that it was 88% and the 16S rRNA showed that it was 96% related to *Bacillus sphaericus* according to the VITEK database and gene bank (Tables 4 and 5).

Isolates 2 (It2), 4 (It4), and 6 (It6): These gram-negative rod and non-spore-forming bacteria were identified as *Acinetobacter* sp., according to morphological, physiological, and biochemical tests (Tables 1-3). Although It2, It4, and It6 belong to the same genus, they do not appear to be the same species. The VITEK system (GNI) showed that they were 37% (It2), 99% (It4), and 80% (It6), and the 16S rRNA showed that they were 97% (It2), 99% (It4), and 97% (It6) related to *Acinetobacter* sp. according to the VITEK database and gene bank (Tables 4 and 5).

Isolate 3 (It3): This gram-negative rod and non-spore-forming bacterium was identified as *Kluyvera cryocrescens*. For this identification, results of the biochemical tests of It3 were compared to bacterial species from the 16S rRNA database (Table 5). The VITEK system also confirmed It3 to belong to the *Kluyvera* genus (Table 4). Because of the diversity of the results of some tests, it was thought that It3 belonged to either species *Kluyvera cryocrescens* or species *K. ascorbata*. The only difference between those species is the presence of lysine decarboxylase (LDC) (*K. cryocrescens*, LDC (-); *K. ascorbata*, LDC (+)) (31). Since It3 was LDH (+), it was inferred that It3 was *Kluyvera cryocrescens*. The other properties of It3 are shown in Tables 1-3. According to results from the VITEK system and16S rRNA, it has 63% and 99% similarity, respectively.

Isolate 5 (It5): This gram-positive rod and non-spore-forming bacterium was identified as *Vagococcus* sp. (Table 1). It did not grow in the VITEK system. The other properties of It5 are shown in Tables 2 and 3. Results from the 16S rRNA showed that it was 99% similar to *Vagococcus carniphilus* and *Vagococcus fluvialis* (Table 5).

Table 1. Morphological, physiological, and biochemical characteristics of bacterial isolates of *I. typographus*.

Tests	Isolates							
	It1	**It2**	**It3**	**It4**	**It5**	**It6**	**It7**	**It8**
Colony color	cream	cream	cream	cream	cream	cream	cream	cream
Shape of bacteria	rod	rod	rod	rod	rod	rod	rod	rod
Gram stain	+	-	-	-	+	-	-	-
Catalase production	+	+	+	+	-	+	+	+
Hydrolysis of urea	-	+	+	+	-	+	+	-
Indole test	-	-	+	-	-	-	+	-
Methyl red test	-	-	-	+	ND	-	+	-
Hydrolysis of starch	-	-	-	-	-	-	-	-
Lysine decarboxylase	ND	ND	-	-	-	ND	-	+
Oxidase	-	-	-	-	-	-	-	-
MCA	-	+	+	+	-	+	+	+
EMB	-	+	+	+	-	+	+	+
MSA	-	-	-	-	-	-	-	-
Mobility	+	-	+	-	+	-	+	+
Growth at 37 °C	+	-	+	+	+	-	+	+
Growth at 40 °C	+	ND	+	+	+	ND	+	+
Growth in 4% NaCl	+	+	+	+	+	+	+	+
Growth in 6.5% NaCl	+	-	+	-	-	-	+	+
Growth in 8.5% NaCl	+	ND	-	ND	ND	ND	+	+

+, Growth Positive; -, Growth Negative; ND, No Data.

Isolate 7 (It7): This gram-negative rod and non-spore-forming bacterium was identified as *Proteus vulgaris*. The other properties of It7 are shown in Tables 1-3. Both the VITEK system (GNI) (Table 4) and the 16S rRNA showed that it was 99% related to *Proteus vulgaris* according to the VITEK database and gene bank (Table 5).

Isolate 8 (It8): This gram-negative rod and non-spore-forming bacterium was identified as *Serratia liquefaciens*. The other properties of It8 are shown in Tables 1-3. Both the VITEK system (GNI) and the 16S rRNA showed that it was 99% related to *S. liquefaciens* according to the VITEK database and gene bank (Tables 4 and 5).

Of the 8 species of bacteria tested against the adults of *I. typographus*, 5 of them caused mortality in healthy adults (Figure). Of these, a significant mortality (53.3%) was only found in adults fed with *S. liquefaciens* (It8) (ANOVA LSD, $P < 0.05$). Some adults treated with other species of bacteria isolated in the study, such as *Bacillus sphaericus* (It1), *Acinetobacter* sp. (It2), *Acinetobacter* sp. (It4), and *P. vulgaris* (It7), also died, but the mortality rate was much lower (13.3%, 16.6%, 16.6%, and 23.3%, respectively).

Table 2. The results of the API 20E test system for bacterial isolates of *I. typographus*.

Tests	Isolates				
	It3	**It4**	**It5**	**It7**	**It8**
β-galactosidase	+	-	-	-	+
Arginine dihydrolase	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	+
Ornithine decarboxylase	+	-	-	-	+
Trisodium citrate	+	-	-	-	+
H_2S (sodium thiosulfate)	-	-	+	+	-
Urease	-	+	-	+	-
L-tryptophan	-	-	-	+	-
Indole	+	-	-	+	-
VP (sodium pyruvate) test	+	-	+	+	+
Gelatinase	-	+	-	+	+
Glucose fermentation	+	+	+	+	+
D-mannitol fermentation	+	-	+	-	+
Inositol fermentation	-	-	-	-	+
D-sorbitol fermentation	-	-	+	-	+
L-rhamnose fermentation	+	-	-	+	-
D-saccharose fermentation	+	-	+	+	+
D-melibiose fermentation	+	+	-	-	+
Amygdaline fermentation	+	-	+	-	+
L-arabinose fermentation	+	+	-	-	+
NO_2	+	-	-	+	+
N_2	-	-	-	-	-

+, Growth Positive; -, Growth Negative
*It1, It2, and It6 did not grow in API 20E.

Table 3. The results of the API 50CH test system for bacterial isolates of *I. typographus*.

Tests	Isolates				
	It3	**It4**	**It5**	**It7**	**It8**
Negative control	-	-	-	-	-
Glycerol	+	-	+	+	+
Erythritol	-	-	-	-	-
D-arabinose	+	-	-	+	-
L-arabinose	+	+	-	-	+
D-ribose	+	+	+	+	+
D-xylose	+	+	-	+	+
L-xylose	-	-	-	-	-
D-adonitol	-	-	-	-	-
Methyl-βD-xylopyranoside	-	-	-	-	-
D-galactose	+	+	-	+	+
D-glucose	+	+	+	+	+
D-fructose	+	-	+	+	+
D-mannose	+	+	+	-	+
L-sorbose	-	-	-	-	-
L-rhamnose	+	-	-	+	-
Dulcitol	-	-	-	-	-
Inositol	-	-	-	-	+
D-mannitol	+	-	+	-	+
D-sorbitol	-	-	+	-	+
Methyl-αD-mannopyranoside	-	-	-	-	-
Methyl-αD-glucopyranoside	+	-	+	+	-
N-acetylglucosamine	+	-	+	+	-
Amygdaline	+	-	+	-	+
Arbutin	+	-	+	-	-
Esculin	+	-	+	-	+
Salicin	+	-	+	-	-
D-cellobiose	+	-	+	-	+
D-maltose	+	-	+	+	+
D-lactose	+	-	+	-	-
D-melibiose	+	+	-	-	+
D-saccharose	+	-	+	+	+
D-trehalose	+	-	+	-	?
Inulin	-	-	-	-	-
D-melezitose	-	-	-	-	+
D-raffinose	+	-	-	-	+
Amylum	-	-	+	-	-
Glycogen	-	-	-	-	-
Xylitol	-	-	-	-	-
Gentiobiose	-	-	+	-	+
D-turanose	+	-	-	+	+
D-lyxose	-	-	-	-	+
D-tagatose	-	-	-	-	-
D-fucose	-	+	-	-	-
L-fucose	+	-	-	-	+
D-arabitol	-	-	-	-	-
L-arabitol	-	-	-	-	-
Potassium gluconate	+	-	-	+	+
Potassium 2-ketogluconate	+	-	-	-	+
Potassium 5-ketogluconate	-	-	-	-	+

+, Growth Positive; -, Growth Negative;
*It1, It2, and It6 did not grow in API 50CH.

Table 4. The results of the VITEK system for bacterial isolates of *I. typographus*.

Isolates	Identifications of Isolates	Identity (%)
It1	*Bacillus sphaericus*	*88*
	Bacillus fusiformis	*88*
It2	*Acinetobacter lwoffii*	*37*
	Acinetobacter junii	*37*
It3	*Kluyvera* sp.	63
It4	*Acinetobacter calcoaceticus*	*99*
	Acinetobacter baumannii	99
		97
It6	*Acinetobacter lwoffii*	*80*
	Acinetobacter junii	*80*
		97
It7	*Proteus vulgaris*	99
It8	*Serratia liquefaciens*	99

It5 did not grow in the VITEK system.

Table 5. Similarity of 16S rRNA gene of isolates to the known bacterial sequences.

Isolates	Identifications of Isolates	GenBank Accession No.	Identity (%)
It1	*Bacillus sphaericus*	DQ870695.1	96
	Bacillus fusiformis	DQ333300.1	*96*
	Bacillus macroides	AJ628749	95
It2	*Acinetobacter haemolyticus*	AM184255.1	97
	Acinetobacter baumannii	CP000521.1	97
	Acinetobacter johnsonii	DQ911549.1	*97*
It3	*Kluyvera cryocrescens*	AF310218	99
	Kluyvera ascorbata	AJ627201	99
	Enterobacter intermedius	AB004747	99
	Enterobacter aerogenes	AB099402	99
	Kluyvera cochleae	AF047187	99
	Citrobacter braakii	AF025368	98
	Citrobacter freundii	AF025365	98
	Klebsiella trevisanii	AF129444	98
	Klebsiella terrigena	AF129442	98
	Klebsiella ornithinolytica	U78182	98
	Klebsiella pneumoniae	AY043391	98
	Morganella morganii	AY043390	98
	Klebsiella planticola	X93215	98
	Klebsiella oxytoca	U78183	*99*

Table 5. (Continued).

Isolates	Identifications of Isolates	GenBank Accession No.	Identity (%)
It4	*Acinetobacter calcoaceticus*	Z93434	99
	Acinetobacter junii	AM184300.1	98
	Acinetobacter baumannii	AY738400.2	97
It5	*Vagococcus carniphilus*	AY179329	99
	Vagococcus fluvialis	Y18098.1	99
It6	*Acinetobacter haemolyticus*	AM184255.1	97
	Acinetobacter johnsonii	DQ911549.1	97
	Acinetobacter baumannii	AY738400.2	97
It7	*Proteus vulgaris*	X07652	99
	Proteus hauseri	DQ885262.1	99
	Proteus mirabilis	AF008582	98
	Proteus penneri	AJ634474	99
	Proteus myxofaciens	AB273746.1	98
It8	*Serratia liquefaciens*	AY243097	99
	Serratia grimesii	AJ233430	99
	Serratia proteamaculans	AY040208	99
	Rahnella aquatilis	AY253920	98
	Aranicola proteolyticus	U93263	99
	Serratia fonticola	AJ233429	98
	Yersinia ruckeri	AF366385	97
	Yersinia kristensenii	AJ627598	98
	Rahnella aquatica	AJ233426	97

Discussion

There has recently been an increasing interest in finding more effective and biologically sound control agents against hazardous insects. Although there have been many biological control studies on *I. typographus*, to date, there has been no study on the isolation and characterization of bacteria from *I. typographus* as potential biological control agents.

Microscopic techniques and 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 a basis for comparison between species. Similarly, the potential sequence analysis of genes coding rRNA and certain proteins affecting the evolution of billions of taxa is documented (32). The introduction of PCR amplification of 16S rRNA genes from bacterial DNA has been used to differentiate bacteria. 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 similarity (33). Thus, in this work, we used the primary structure of the 16S rRNA for identification of isolates from *I. typographus* in addition to microscopic techniques and physiological and biochemical tests.

In this work, we isolated 8 bacteria and identified them. One was *Bacillus sphaericus* (It1), which has also been isolated from hazelnut pests, *Anoplus roboris* (Col.: Curculionidae) (34) and *Melolontha*

melolontha (Col.: Scarabaeidae) (10). There has also been some information in the literature that *Acinetobacter* sp. (It2, It4 and It6) has frequently been isolated from arthropods (35), *Oberea linearis* (36), and *Melolontha melolontha* (10). However, *Kluyvera cryocrescens*, coded as It3 in this work, is here first reported as isolated from insects. Isolate 5 showed some features of both *V. carniphilus* and *V. fluvialis*. This bacterium was thus identified as *Vagococcus* sp. According to the morphological, biochemical, and molecular characteristics of It7, it was identified as *Proteus vulgaris*. Another isolate of *Proteus vulgaris* has been isolated from *Cydia pomonella* (37). *S. liquefaciens* (It8) is also well known in microbiology, first as *Enterobacter liquefaciens* and then as *S. liquefaciens*. Isolates have frequently come from water, plants, insects, food, and other environmental sources (38). It8, also, has a highly insecticidal activity; it is thought that this is due to *S. liquefaciens*' ability to degrade chitin, by which it has the activity of chitinase (unpublished results). This supposition is matched by some information in the literature about chitinase being produced by some bacteria such as *Serratia marcescens* (39).

Of the 8 species of bacteria tested against the adults of *I. typographus*, 5 caused mortality in healthy adults (Figure). Of these, a significant mortality (53.3%) was only found in adults fed with *S. liquefaciens* (It8) (ANOVA LSD, $P < 0.05$). Although *Bacillus sphaericus* (It1), *Acinetobacter* sp. (It2), *Acinetobacter* sp. (It4), and *P. vulgaris* (It7) also caused death, they did not significantly differ from the PBS control. They also showed very different frequencies of isolation for each bacterial species (Table 6). In addition, the number of bacteria in the adults was determined by counting the number of colonies on the plates that had been inoculated with the diluted bacterial suspensions. The total number of bacteria was found to be $(4.6 \pm 0.35) \times 10^3$ cfu/adult (n = 15) in *I. typographus*.

Wegensteiner and Weiser performed several studies about pathogens such as gregarian pathogens, viruses, and microspores. Several pathogens are reported to occur in *Ips typographus* (9,40-43).

However, bacteria-insect interaction is not only pathogenic, but also symbiotic. Symbiotic bacteria are ubiquitously located in insect guts, with these symbioses ranging from pathogenic to mutualistic

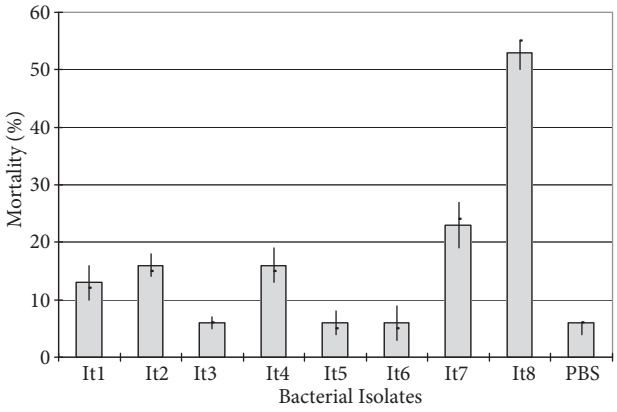

Figure. Results of insecticidal effects of bacterial isolates on *Ips typographus* adults 10 days after exposure. Bacterial isolates: It1, *Bacillus sphaericus*; It2, *Acinetobacter* sp.; It3, *Kluyvera cryocrescens*; It4, *Acinetobacter* sp.; It5, *Vagococcus* sp.; It6, *Acinetobacter* sp.; It7, *Proteus vulgaris*; It8, *Serratia liquefaciens*; PBS, negative control. Vertical bars represent standard deviation. ANOVA ($F = 52.678$; $P < 0.05$) LSD test.

Table 6. The frequency of isolation or identification of bacteria from *I. typographus*.

Isolates	**Frequency (%)**
It1	1 (4.7)
It2	2 (9.52)
It3	3 (14.28)
It4	3 (14.28)
It5	2 (9.52)
It6	2 (9.52)
It7	4 (19.04)
It8	4 (19.04)

and from facultative to obligate (13). Determining the symbiotic bacteria in insect species can allow for the development of new approaches to biological control (44). Therefore, many scientists have investigated the bacterial flora of harmful insects (20,34,45).Consequently, it was determined that *S. liquefaciens* in particular may be used as a biological control agent against *I. typographus* adults. Chitinase activities may play key roles in the virulence of some pathogens that infect insects via the peritrophic membrane, including the malarial parasite *Plasmodium gallinaceum* (46), the trypanosome

Leishmania (47), the nematode *Brugia malayi* (48), and the *Autographa californica* nucleopolyhedrovirus (49). Bacterial chitinase and its gene, in the case of the chitinase of *S. liquefaciens*, are now available for biopesticidal applications in integrated pest management programs. There have been reports that addition of commercial chitinase preparations (50), crude chitinase preparations from *Bacillus circulans* (51), and even of chitinolytic bacteria extracted from the insect's gut (52), enhance the insecticidal activity of *B. thuringiensis*. Future studies will be conducted with the aim of finding a biological control agent against this hazardous insect, using this bacterium or other newly isolated agents. The present study has contributed significantly to the literature on bacterial isolates of the eight-toothed spruce bark beetle.

Conclusions

This is the first detailed investigation of bacteria from *I. typographus*, and some of them appear to be promising for use against this pest. However, further bioassay studies will be needed to prove this. Because the injection of bacterial culture into the bark was very difficult, insects were exposed to less fatal bacterial cultures. Therefore, the mortality rate was lower than expected. If more effective methods are used for bioassay on adult specimens, this rate would probably increase effectively.

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