J. Microbiol. Biotechnol. (2007), 17(6), 968–978

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Identification of Two Entomopathogenic Bacteria from a Nematode Pathogenic to the Oriental Beetle, *Blitopertha orientalis*

YI, YOUNG-KEUN[1], HAE WOONG PARK[2], SONY SHRESTHA[1], JIAE SEO[1], YONG OOK KIM[2], CHUL SOO SHIN[2], AND YONGGYUN KIM[1]*

[1]*Department of Bioresource Sciences, College of Natural Sciences, Andong National University, Andong 760-749, Korea*
[2]*Department of Biotechnology, College of Engineering, Yonsei University, Seoul 120-749, Korea*

Received: December 28, 2006
Accepted: February 21, 2007

Abstract A pathogenic nematode, *Butlerius* sp., was isolated from Oriental beetle, *Blitopertha orientalis*. The infective juveniles exhibited dose- as well as time-dependent entomopathogenicity on the larvae of *B. orientalis*. Two bacterial species, *Providencia vermicola* (KACC 91278) and *Flavobacterium* sp. (KACC 91279), were isolated from the infective juveniles and identified. *P. vermicola* outnumbered *Flavobacterium* sp. in the nematode host, in which the colony density of *P. vermicola* was found to be 21 times higher than that of *Flavobacterium* sp. However, when the two bacterial species were cocultured in culture media without the nematode host, they showed similar growth rates. Both bacteria induced significant entomopathogenicity against *Spodoptera exigua* larvae infesting economically important vegetable crops, where *P. vermicola* was more potent than *Flavobacterium* sp.

Keywords: Entomopathogenicity, *Providencia vermicola*, *Flavobacterium*, *Butlerius*, ITS, 16S rDNA

Entomopathogenic nematodes include two families of Heterorhabditidae and Steinernematidae, which possess species-specific bacteria for completing successful parasitic life cycles [3, 6]. The symbiotic bacteria in a vesicle are usually located in the gut lumen of the nematode host [36]. The nematode enters a target insect, in which it promotes the release of the bacteria into the hemocoel. The released bacteria depress insect immune reactions by inhibiting biosynthesis of eicosanoids that play an important role in mediating immune signal to immune effectors [32]. In this immunosuppressive host condition, the bacteria multiply and kill the host insect by septicemia [14, 40]. The symbiotic nematode then resumes development and reproduction in the insect cadaver. The multiplied infective juveniles egress from the insect host and look for other target hosts [30].

Entomopathogenic nematodes have been used as commercial biopesticides [29]. However, their susceptibility to UV and desiccation restricts their use in specific target insect pests infesting in greenhouse or underground conditions [38]. On the other hand, their symbiotic bacteria have been considered to be a novel control agent against insect and microbial pests [10, 35]. Because of their antimicrobial activity, various novel antimicrobial compounds have been identified and applied to control plant pathogens [25, 55]. Furthermore, their high insecticidal potency is used to develop an integrated biological agent mixture with *Bacillus thuringiensis*, because the bacteria alone cannot infect insect hemocoel, where the bacteria express their insecticidal activity [26–28].

A current surveillance for new entomopathogenic nematodes isolated a new group of entomopathogenic nematode, which was not classified into either Steinernematidae or Heterorhabditidae. This research reports a novel entomopathogenic nematode and its associating bacteria. The identified bacteria exhibited significant entomopathogenicity on *Spodoptera exigua*, an insect pest causing serious economic damage on vegetable crops [8, 31, 50].

Materials and Methods

Nematode Collection

An entomopathogenic nematode was isolated from Oriental beetle, *Blitopertha orientalis*, collected at Bokwang Phoenix Park (Pyungchang, Kangwon, Korea). After infecting *B. orientalis*, the resulting infective juveniles were collected by an improved Baermann funnel trap method [33]. The third instar larvae of *B. orientalis* collected from the field

*Corresponding author
Phone: 82-54-820-5638; Fax: 82-54-823-1628;
E-mail: hosanna@andong.ac.kr

were used for entomopathogenic experiment. *Sp. exigua* larvae were fed and reared on an artificial diet [18] at 25±1°C. Second and fifth instar larvae were used for oral infection and injection bioassays, respectively.

Bacteria Isolation from Nematodes

About 50 µl of infective juveniles (IJs) were surface-sterilized for 30 min with 0.06% sodium hypochloride containing 250 ppm of streptomycin, and this was followed by three rinses in sterile Ringer's solution [23]. The sterilized nematodes were homogenized in the Ringer's solution, diluted with an equal volume of sterilized water, and streaked on tryptic soy agar (TSA) (Difco, U.S.A.) plates. These plates were left for bacterial growth in an anaerobic incubator at 25°C for 2 days. Two types of bacterial colonies showing different colors were isolated and further cultured in TSB for preparing freeze stocks, which were used for subsequent experiments in order to avoid any genetic change due to successive cultures.

Biochemical Characters of Nematode-associated Bacteria

Carbon source utility of the bacteria was analyzed by a microbial identification system (Biolog, U.S.A.) using a GN microplate. Fatty acid composition of the bacterial cell wall was analyzed by a gas chromatography (Agilent 6890 series, U.S.A.). Other biochemical characters were examined according to the procedures of Benson [4] and Schaad [45].

Sequence Analysis of Internal Transcribed Spacer (ITS) Region of Nematode Ribosomal DNA (rDNA)

About 0.5 g of IJs were homogenized and genomic DNA was isolated from the homogenate by incubation with proteinase K, followed by phenol/chloroform extraction and ethanol precipitation [44]. For polymerase chain reaction (PCR) amplification and sequencing of the ITS, primers were designed by the method of Vrain *et al.* [51]: forward and reverse primers were 5'-TTGAT TACGT CCCTG CCCTT T-3' and 5'-TTTCA CTCGC CGTTA CTAAG G-3', respectively. All tubes were kept on ice, and the following PCR mixture was added to each tube: 5 µl of 10×PCR buffer, 5 µl of dNTP mixture (2.5 mM each), 2 µl of 25 pmol forward primer, 2 µl of 25 pmol reverse primer, 1 µl of Taq polymerase (5 U/µl), 1 µl of template DNA, and 34 µl of distilled water. PCR (PTC-100 MJ Research, Minnesota, U.S A.) conditions were 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The PCR product was cloned into pGEM-T vector (Promega, Madison, U.S.A.). The inserts were bidirectionally sequenced. All sequencings were preformed by a DNA sequencing company (Microgen, Daejon, Korea). The sequencing data were aligned and corrected, if necessary, by the DNAstar program (Version 5.01, DNAstar Inc, Madison, U.S.A.). The resulting ITS sequence was deposited to NCBI with the accession number of EF192135.

16S rDNA Sequence Analysis of Bacteria

Total genomic DNAs were extracted from cultured bacterial isolates [44]. Universal polymerase chain reaction (PCR) primers [11, 34 55] were used to amplify the 16S rDNA (approximately 1.6 kb), where the forward and reverse primers were 5'-GAAGA GTTTG ATCAT GGCTC-3' and 5'-AAGGA GGTGA TCCAG CCGCA-3', respectively. All PCR conditions and sequence analysis were the same as described for the nematode ITS analysis. In this study, two bacterial isolates were analyzed, where nucleotide sequences at 16S rDNA of white and yellow bacteria were deposited to NCBI with accession numbers of EF192136 and EF192137, respectively.

Electron Microscopy

The bacterial suspension was put on formvar-coated grids for 15 sec and then stained with 2% uranyl acetate for

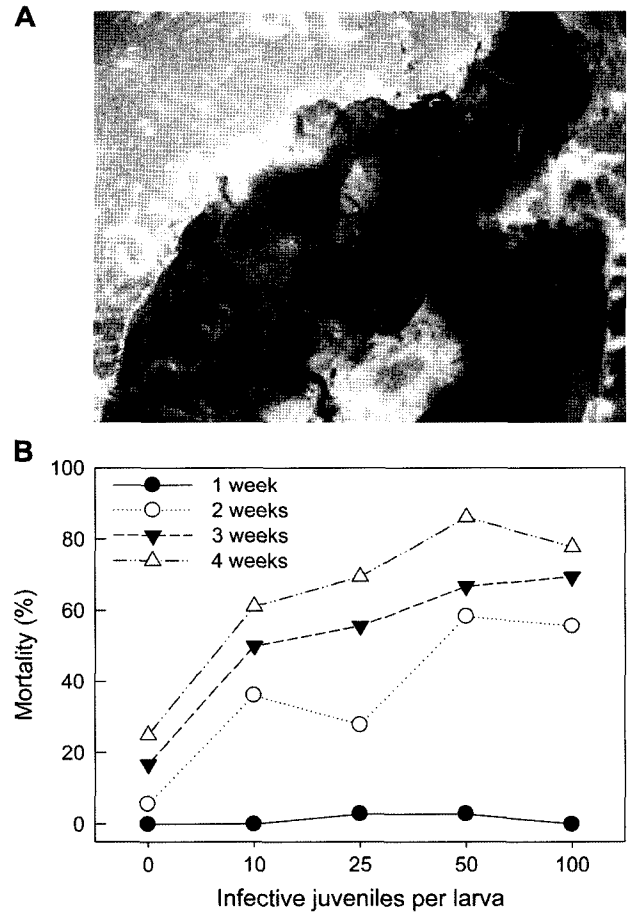

Fig. 1. An entomopathogenic nematode isolate from a field-collected Oriental beetle, *Blitopertha orientalis*.
(**A**) The nematodes developing in the infected insects and (**B**) their pathogenicity to *B. orientalis* larvae.

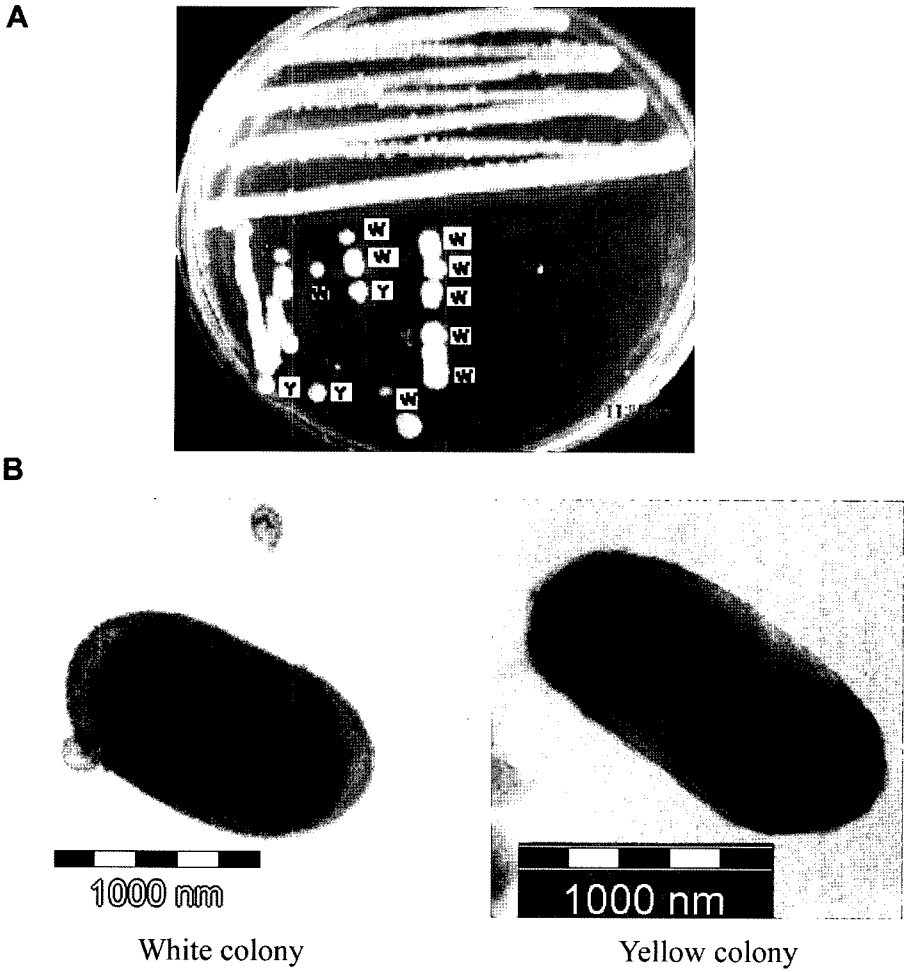

Fig. 2. Bacteria isolated from *Butlerius* sp. cultured on TSA in anaerobic condition.
A. Two different colored bacteria were found: white (W) or yellow (Y) colony. **B.** Each bacterium was observed by transmission electron microscopy. The white colony bacterium contained peritrichous flagella, but the yellow colony bacterium did not.

Table 1. Characters for genus identification of a white colony bacterium isolated from an entomopathogenic nematode, *Butlerius* sp.

Characters	White colony	*Providencia*[a]
Gram reaction	-	-
Facultative anaerobic	+	+
Cell morphology	Rod	Rod
Flagella	Peritrichous	Peritrichous
Colony color	White	White
Oxidase	-	-
Catalase	-	-
Nitrate reduction	+	+
Phenylamine deaminase	+	+
Indole production	+	+
Methyl red	+	+
Gelatin hydrolase	-	-
Arabinose	-	-
Gas from glucose	-	-

[a]The characteristics cited from Penner [41] and Brenner [7]. Symbols: "+" when more than 90% of strains are positive and "-" when less than 10% of strains are positive.

25 min. The stained grids were located on a transmission electron microscope (LEO 906E, Oberkochen, Germany).

Nematode and Bacterial Pathogenic Assay

Insecticidal activities of nematodes were determined in a 6-well plate by placing *B. orientalis* larvae separately with different concentrations of IJs on moist filter paper. Each IJ concentration consisted of 36 test larvae. Mortality was assessed every day and analyzed on a weekly basis. The median lethal concentration (LC_{50}) of nematodes was estimated by Probit analysis [42].

Assessment of bacterial pathogenicity was performed by both *in vivo* injection and oral feeding methods, where *Sp. exigua* larvae were used as test insects. For the injection assay, 5 µl of the bacterial suspension (10^3–10^{10} CFU/ml in Ringer's solution) was injected into the hemocoel of the larvae by a 10-µl Hamilton microsyringe (Hamilton, Nevada, U.S.A.), where Ringer's solution served as the control. Each concentration was tested using

nine insect larvae with three independent replications. Mortality was assessed at every 24 h after treatment. For oral infection, the artificial diet was cut into small pieces (about 1 cm^3) and soaked into predetermined concentrations (2.0×10^3–2.0×10^{10} CFU/ml in Ringer's solution) of bacterial suspension for 5 min. After drying in the dark for about 10 min, the treated diet was given to the test larvae. Each bacterial concentration was fed to 15 larvae and run independently three times. Mortality was determined at every 24 h after treatment. Larvae were considered dead or dying if they did not move in a coordinated manner when prodded with a blunt probe. Both pathogenicity results were analyzed using Probit analysis.

RESULTS AND DISCUSSION

Isolation of Entomopathogenic Nematode

Several types of entomopathogenic nematodes were obtained from field-collected grubs in golf courses in Korea, of which an entomopathogenic nematode isolate exhibiting high potency in a laboratory test against *B. orientalis* larvae (Fig. 1A) was chosen for further analysis. Significant pathogenicity (Fig. 1B) was observed at 2 weeks (LC_{50}= 69.9 IJs) after IJ treatment and increased with time (LC_{50}= 26.6 IJs at 3 weeks and LC_{50}=9.6 IJs at 4 weeks). At 100 IJ treatment, the median lethal time (LT_{50}) was 16.4 days. The pathogenicity did not enhance even at higher IJ concentrations (data not shown). Though this nematode isolate clearly showed entomopathogenicity against *B. orientalis* because of its infection and growth in the insect host cadaver, their pathogenic potency appeared to be very low, compared with typical entomopathogenic nematodes, *Steinernema glaseri* and *Heterorhabditis* spp. against grubs, which usually showed 80% or higher mortalities in 3 days after treatment [12]. Specifically, these typical entomopathogenic nematodes are well adapted to parasitize sedentary subterranean scarabaeid larvae, partly owing to their cruise foraging strategy [19] and ability to overcome host defenses [54]. Thus, our nematode isolate may differ in these entomopathogenic characters compared with typical potent entomopathogenic nematodes.

There were also significant differences between the nematode isolate and typical entomopathogenic nematodes in major morphological characters such as spicule length and gubernaculum length (data not shown). To identify the nematode isolate, we used ITS sequence data because of its usefulness for species identification of typical entomopathogenic nematodes [1, 5, 49]. Using universal primers, two ITS regions were obtained along with partial 18S rDNA, full 5.8S rDNA, and partial 28S rDNA sequences. This sequence showed the highest homology (nucleotide identity=92%; E value=$5e^{-59}$) with that of *Butlerius* sp. MJ-2005, which was isolated from soil in South Africa and characterized as an entomophilic Pseudodiplogasterid nematode (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=74132450). Although there was some similarity to *Heterorhabditis* spp. in this DNA sequence, the lack of typical *Heterorhabditis*-associated bacteria as well as morphological difference did not support its identification. Our molecular identification suggests a novel *Butlerius* sp. because no known *Butlerius* spp. have been reported to be entomopathogenic. *Butlerius* spp. have usually been regarded as a casual associate of insect grubs normally existing in the soil and observed to be predatory on other nematodes [24]. To identify the species, a detailed taxonomic research has to be performed. In this study, this new nematode isolate was named as *Butlerius* sp.

Identification of Two Bacteria Associated with Entomopathogenic Nematode, *Butlerius* sp.

The entomopathogenicity showed by the nematode isolate suggested that there would be symbiotic bacteria in the nematode, which play a significant role in the insecticidal activity. Two different colored bacteria were isolated from the nematode (Fig. 2A). White-colored bacteria showed

Table 2. Characters for genus identification of a white colony bacterium isolated from an entomopathogenic nematode, *Butlerius* sp.

Characters	Yellow colony	Similar taxa[a]							
		Fla	CyFl	AgXa	EnVi	Kin	AcBo	BrNe	Cory
Gram reaction	–	–							+
Gliding motility	–	–	+						
Functional flagella	–	–		+					
Fermentative in glucose O-F medium	–	–			+				
Catalase production	+	+				–			
Oxidase production	+	+					–		
Penicillin (1.0 u/ml) susceptibility	–	–						+	

[a]Abbreviation: "Fla" for *Flavobacterium*; "CyFl" for *Cytophaga* and *Flexibacter*; "AgXa" for *Agrobacterium*, *Alcaligenes*, *Alteromonas*, *Bordotella bronchiseptica*, *Pseudomonas*, and *Xanthomonas*; "EnVi" for Enterobacteriaceae, Pasteurellaceae and Vibrionaceas; "Kin" for *Kingella*; "AcBo" for *Acinetobacter* and *Bodetella parapertussis*; "BrNe" for *Branhamella catarrhelis*, *Moraxella*, and *Neisseria*; "Cory" for Coryneforms.
[b]Symbols: "+" when more than 90% of strains are positive and "–" when less than 10% of strains are positive.

significant motility under microscopy and had peritrichous flagella, but the yellow-colored bacteria did not show any motility and flagella (Fig. 2B).

To identify the bacteria, 16S rDNA sequences of both bacteria were determined. Based on these 16S rDNA sequences, the white-colored colony showed high homology (nucleotide identity=98%; E value=0.0) with *Providencia vermicola*, whereas the yellow-colored colony did so with *Flavobacterium breve* (nucleotide identity=98%; E value=0.0). This molecular identification was consistently supported by two different microbial identification systems, in which both fatty acid composition and Biolog results indicated that the white-

Table 3. Characters for species identification of a white colony bacterium isolated from an entomopathogenic nematode, *Butlerius* sp., based on Biolog GN substrate reactions.

Characters	White isolate	Responses of *Providencia* species[a]					
		vermicola	*rettgeri*	*stewartii*	*rustigiani*	*hemibachea*	*alcalifaciens*
Dextrin	+	–	–	–	w	–	–
Tween 40	+	–	–	+	–	–	–
Tween 80	+	+	+	+	–	w	+
L-Arabinose	–	+	–	–	–	–	–
L-Erythritol	+	+	–	–	–	–	–
D-Fructose	+	+	–	–	–	–	–
D-Galactose	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+
Myo-inositol	+	+	+	+	+	–	–
Methyl-D-glucoside	+	–	w	–	–	–	–
D-Psicose	+	+	–	–		–	–
Monomethyl succinate	+	v(–)	–	–	–	+	+
Acetic acid	+	+	w	w	–	+	+
cis-Aconitic acid	+	+	–	+	–	+	+
Citric acid	+	+	–	+	–	+	+
Formic acid	+	v(+)	–	–	–	+	–
D-Glucosamine	–	–	–	–	–	–	–
p-Hydroxyphenylacetic acid	+	–	–	+	+	–	–
α-Ketoglutaric acid	+	+	–	+	–	+	+
DL-Lactic acid	+	v(+)	–	–	w	–	–
Glucuronamide	+	v(+)	–	–	–	–	–
DL-Alanine	+	+	–	+	–	–	–
L-Alanyl glycine	+	+	+	+	–	–	–
Glycil-L-aspartic acid	+	+	+	–	–	–	–
Glycil-L-glutamic acid	+	+	+	+	w	+	+
L-Histidine	+	+	+	+	w	+	+
L-Hydroxyproline	+	+	+	+	–	+	+
L-Leucine	+	–	–	–	–	w	–
L-Pyroglutamic acid	+	+	+	+	–	+	w
D-Serine	+	+	+	+	+	+	w
L-Threonine	+	w	–	–	–	w	–
Carnitine	+	–	–	w	–	–	–
Inosine	+	+	+	+	–	+	–
Uridine	+	+	+	+	+	+	w
Thymidine	+	+	+	+	–	+	w
Phenylethylamine	+	–	–	+	–	–	–
Glycerol	+	+	+	+	–	+	+
Glycerol phosphate	+	+	+	+	w	+	+
Glucose-1-phosphate	+	+	+	+	+	+	w
Glucose-6-phosphate	+	+	+	+	–	+	w

[a]The characteristics of genus *Providencia* were cited from Somvanihs *et al.* [46]. Symbols: "+" when more than 90% of strains are positive, "–" when less than 10% of strains are positive, "v(+)" when more than 50% of strains are positive, "v(–)" when more than 50% of strains are negative, and "w" at weak reactions.

Table 4. Characters for species identification of a yellow colony bacterium isolated from an entomopathogenic nematode, *Butlerius* sp.

Characters	Yellow colony	Responses of *Flavobacterium* species[1]								
		aquatile	*balustinum*	*branchlophile*	*breve*	*greum*	*indologenes*	*meningosepticum*	*odoratum*	*thalpophilum*
Cell morphology	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod
Gram reaction	–	–	–	–	–	–	–	–	–	–
Motility	–	–	–	–	–	–	–	–	–	–
Aerobic	+	+	+	+	+	+	+	+	+	+
Growth at 37°C	+	+	+	+	+	+	+	+	+	+
Growth at 45°C	–	–	–	–	–	–	–	–	–	+
Yellow pigment	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Acid from carbohydrate										
Glucose	–	+	+	+	d	+	+	d	–	+
Arabinose	–	–	–	–	–	d	–	–	–	+
Cellobiose	+	–	–	w	–	–	–	–	–	+
Ethanol	–	–	+		–	–	d	d	–	–
Lactose	–	+	–	–	–	–	–	d	–	+
Mannitol	–	–	–	–	–	–	d	d	–	–
Maltose	–	+	–	+	d	+	+	+	–	+
Raffinose	–	–	–	w	–	–	–	–	–	+
Salicin	–	–	–	–	–	–	–	–	–	+
Sucrose	–	+	–	+	–	–	–	–	–	+
Rhamnose	–	–	–	–	–	–	–	–	–	+
Trehalose	+	–	–	+	–	+	+	d	–	+
Xylose	–	–	–	–	–	d	–	–	–	+
Adonitol	–	–	–	–	–	–	–	–	–	+
Glycerol	–	–	–		–	d		d	–	+
Gas from carbohydrate										
Indole	w	–	+	–	+	+	+	d	–	
Nitrate reduction	–		+	–	–		d	–	–	+
Nitrite reduction	–		–		–	d	d	d	+	–
Starch hydrolysis	–		–	+	–		+	–	–	–
Gelatinase	+	–	–	–	–			d	–	+

[a]The characteristics cited from Holt *et al.* [22]. Symbols: "+" when more than 90% of strains are positive, "–" when less than 10% of strains are positive, "d" when 11–89% of strains are positive, and "w" at weak reactions.

colored colony shared the highest homology with *Providencia* sp., and the yellow-colored colony did so with *Flavobacterium* sp. (data not shown).

Further identification for morphological and biochemical characters followed were as described in Bergey's manual [7, 21, 22, 41]. Two bacterial isolates were different in major bacterial characters such as oxidase, catalase, and nitrate reduction, in which white- and yellow-colored colonies were similar to genus *Providencia* (Table 1) and genus *Flavobacterium* (Table 2), respectively. To identify the species, more than 30 different biochemical characters of each bacterial isolate were compared with those of known species in each corresponding genus group (Tables 3, 4). The white-colored colony showed the highest homology (82.5%) with *P. vermicola*. On the other hand, the yellow-colored colony showed the highest homology (75%) with *F. breve*, *F. greum*, and *F. odoratum*. These results indicate that both bacterial colonies are clearly different from each other and that the white-colored colony is *P. vermicola* and the yellow-colored is *Flavobacterium* sp.

Entomopathogenic bacteria symbiotic to entomopathogenic nematodes include the species of *Xenorhabdus* and *Photorhabdus*, which are associated with Steinernematidae and Heterorhabditidae, respectively [17]. Because of mutualism between the nematode and bacterium, each nematode species has a specific association with only one bacterial species, although the bacterial species may be associated with more than one nematode species [30]. This specific interaction was explained by an observation of the bacterial initial colonization in the nematode gut with monoclonal

origin in the *X. nematophila-St. carpocapsae* complex [37]. However, there are a number of reports of other bacteria, such as *Pseudomonas aeruginosa*, *Enterococcus* sp., *Ochrobactum* spp., and *Acinetobacter* spp., being isolated from the gut and surface of the nematode and from the infected insect host [2, 53]. These studies concluded that the non-*Xenorhabdus* or *Photorhabdus* bacteria do not play a significant role in nematode infection. *P. vermicola* has been initially isolated from *St. thermophilum*, which also contains another bacterium, *X. indica* [46, 47]. Though *X. indica* exhibited strong antibiotic activity as reported for most of the described *Xenorhabdus* species, it did not show any antibiotic activity against *P. vermicola* [47]. However, any physiological role of *P. vermicola* was not known in the infection of *St. thermophilum* or in the interaction with *X. indica*. This study tested for entomopathogenic activity of *P. vermicola* and its interaction with another bacterial inhabitant, *Flavobacterium* sp., of the nematode (see below).

Entomopathogenicity of Nematode-associated Bacteria

Two bacterial isolates were maintained in a nematode host. Direct counts of the isolated colonies existing in the nematode showed that about 21 times more white colonies were possessed by the nematode host compared with yellow colonies (Fig. 3A). However, when these two bacteria were cultured in TSB medium without nematode host, both were able to propagate together and showed similar bacterial population densities (Fig. 3B). Like other entomopathogenic bacteria [46], both bacterial isolates showed high antibacterial activities against the intestinal bacterial population of *B. orientalis* (data not shown). This suggests that these two bacteria can produce their own

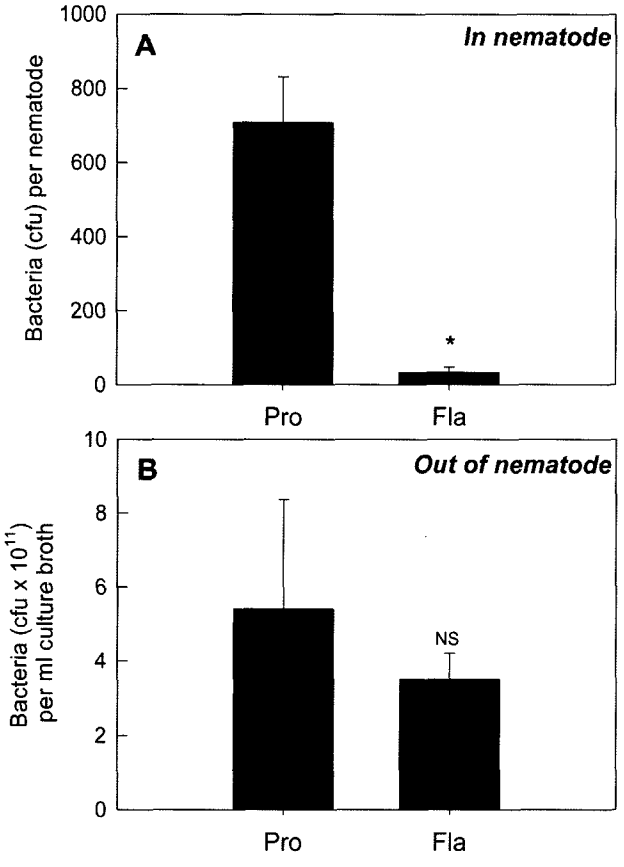

Fig. 3. Population densities of two bacterial species associated with an entomopathogenic *Butlerius* sp., in which "Pro" and "Fla" represent *Providencia vermicola* and *Flavobacterium* sp., respectively.

A. Two types of bacteria were isolated from the nematode and counted. **B.** With the same inoculation concentration (2.7×10^7 CFU), the two bacteria were cultured together in 1 l of TSB at 25°C for 12 h, at which the bacterial growth reached a stationary phase. Three measurements were independently replicated. The asterisk above the error bar indicates significant difference of means between the two bacteria at Type I error=0.05 (LSD test); "NS" represents no significant difference.

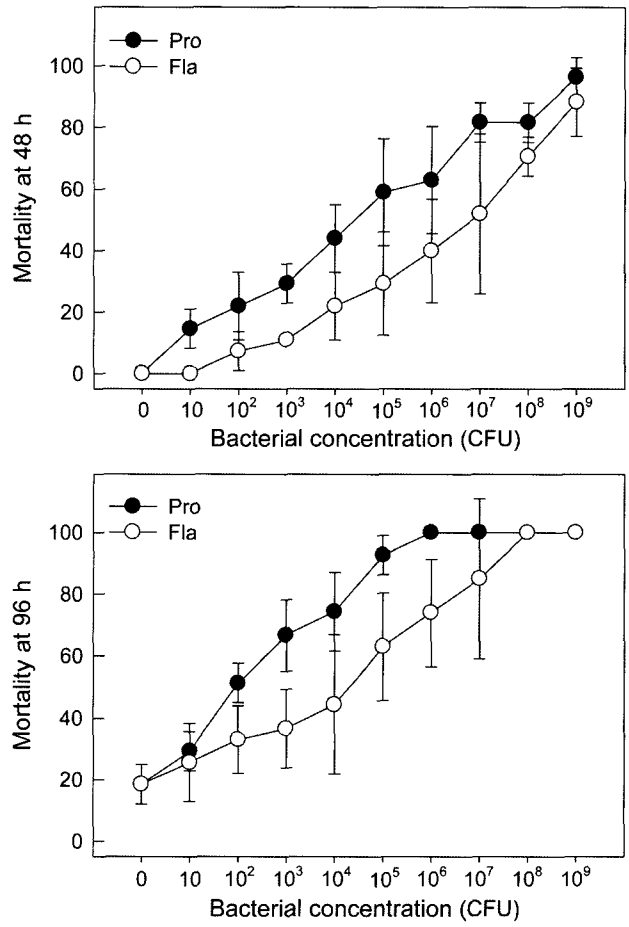

Fig. 4. Pathogenicity of *Providencia vermicola* (Pro) and *Flavobacterium* sp. (Fla) to the fifth instar larvae of *Spodoptera exigua*.

The larvae injected intrahemocoelically with different colony forming units (CFU) of the bacteria were kept at 25°C for 48 h and 96 h for mortality assessment. Each measurement consisted of 9 larvae with three independent replications.

Table 5. Median lethal doses (LD_{50}) of two bacteria against *Spodoptera exigua*, in which the fifth instar larvae were hemocoelically injected with different concentrations of bacteria. Each concentration consisted of 9 larvae and was independently replicated three times.

Bacterial treatment[a]	LD_{50} (CFU)[b]	95% CI	Slope±SE	c^b	df	*P*
		48 h after treatment				
Pro	1.40×10^5 b	3.24×10^4–5.27×10^5	0.37±0.05	2.75	7	0.0925
Fla	8.37×10^6 a	2.27×10^6–3.16×10^7	0.47±0.08	1.81	5	0.1257
		96 h after treatment				
Pro	5.43×10^2 b	1.11×10^2–1.75×10^3	0.55±0.10	1.96	4	0.2560
Fla	9.92×10^4 a	2.37×10^4–3.24×10^5	0.53±0.09	2.79	6	0.1652

[a]"Pro" and "Fla" represent *Providencia vermicola* and *Flavobacterium* sp., respectively.
[b]Different letters following a lethal dose indicate significant difference on the basis of nonoverlap of 95% confidence interval (CI).

antibacterial substances, which are not effective to inhibit growth of each other. It is interesting that the host nematode appears to favor *P. vermicola* for its internal colonization. This was supported by an observation of biased population density of *P. vermicola* when two the bacteria were cultured with the nematode host in TSB media (data not shown). These results suggest that the host nematode plays an important role in maintaining a specific ratio between these two bacterial population densities during nematode multiplication. Though their interaction is not clear at present, it appears that the nematode needs both bacterial symbionts for its successful parasitic life in target insect hosts, in which *P. vermicola* may play a significant role in killing the insect host and converting insect cadaver into a favorable environment for nematode development, with some role of *Flavobacterium* sp. Based on this speculation, we analyzed the entomopathogenicity of both bacteria.

Both bacterial species showed significant entomopathogenic activities against *Sp. exigua* larvae. When the bacteria were injected into insect hemocoel, both bacteria showed significant pathogenicity in high doses at 24 h after treatment (data not shown) and at most doses after 48 h (Fig. 4). However, there was a significant difference in the entomopathogenicity between the two bacteria (Table 5). Based on LD_{50}, *P. vermicola* was ca. 60 and 183 times more potent at 48 h and 96 h after treatment, respectively. Upon experimental oral feeding of these bacteria to *S. exigua* larvae, both bacteria induced pathogenicity, but *P. vermicola* was more acute than *Flavobacterium* sp. (Fig. 5). There was no synergistic effect on the entomopathogenicity between the two bacteria because of no significant increase of mixture treatment in the pathogenicity compared with *P. vermicola* treatment alone (Table 6). A laboratory test of the host nematode, *Butlerius* sp., showed pathogenicity against *Sp. exigua* (data not shown), suggesting a significant role of these symbiotic bacteria in the entomopathogenic progress.

The entomopathogenic ability of the symbiotic bacteria is necessary for host nematode development, and is achieved by insect immunosuppression in at least three successive levels usually found in several bacteria-nematode complexes [13]. As a passive way, such as an evasion from immunocompetent cells, Dunphy and Webster [15, 16] found that surface lipid components of the infective juveniles of *Steinernema carpocapsae*, or outer membrane carbohydrates and proteins of the symbiotic bacteria *X. nematophila*, could evade

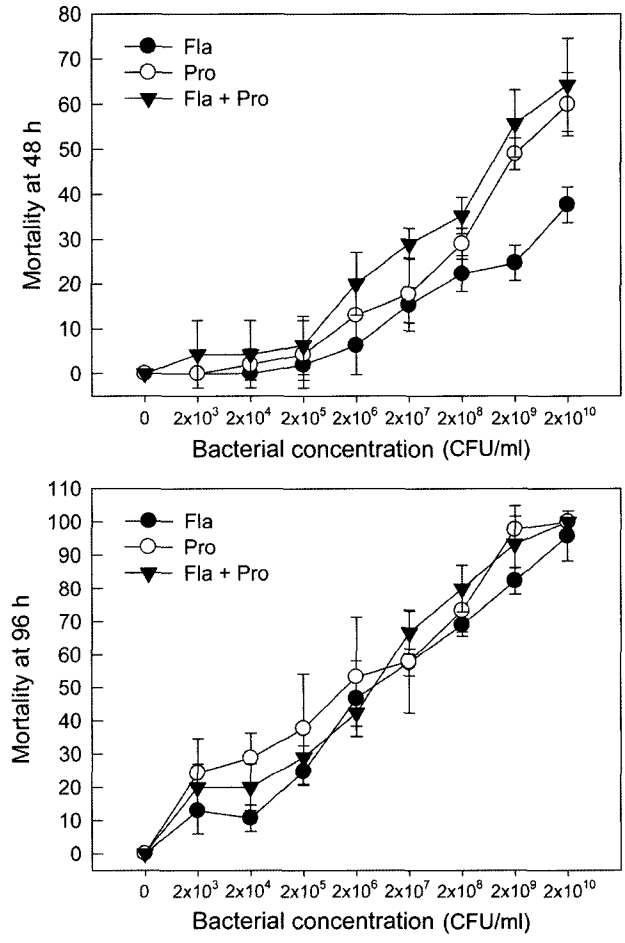

Fig. 5. Pathogenicity of *Providencia vermicola* (Pro) and *Flavobacterium* sp. (Fla) to the second instar larvae of *Spodoptera exigua*.
The larvae were fed with diets soaked in bacterial solutions containing different colony forming units (CFU) for 24 h. The mortality was measured at 48 h and 96 h since the bacterial feeding. Each measurement consisted of 15 larvae with three independent replications.

Table 6. Median lethal concentrations (LC_{50}) of two bacteria on *Spodoptera exigua*, in which the second instar larvae were fed different concentrations of bacteria. Each concentration consisted of 15 larvae and was independently replicated three times.

Bacterial treatment[a]	LC_{50} (CFU/ml)	95% CI	Slope±SE	c[b]	df	P
		48 h after treatment				
Pro	3.45×10^{9} b	1.08×10^{9}–1.67×10^{10}	0.38±0.05	3.02	6	0.1940
Fla	1.31×10^{11} a	1.77×10^{10}–5.02×10^{12}	0.32±0.05	2.47	5	0.2196
Pro+Fla	1.25×10^{9} b	4.02×10^{8}–5.50×10^{9}	0.35±0.04	1.33	6	0.0682
		96 h after treatment				
Pro	1.50×10^{7} a	5.54×10^{6}–3.46×10^{7}	0.64±0.08	7.28	6	0.7040
Fla	3.47×10^{7} a	1.16×10^{7}–8.95×10^{7}	0.52±0.07	3.71	4	0.5533
Pro+Fla	9.63×10^{6} a	3.55×10^{6}–2.21×10^{7}	0.64±0.08	2.49	6	0.1308

[a]"Pro" and "Fla" represent *Providencia vermicola* and *Flavobacterium* sp., respectively.
[b]Different letters following a lethal concentration indicate significant difference on the basis of nonoverlap of 95% confidence interval (CI).

attachment of the hemocytes of *Galleria mellonella* probably because of a lack of nonself recognition. Phospholipase A_2 (PLA_2) catalyzes the committed step of biosynthesis of eicosanoids that play an important role in mediating immunity, especially in response to bacterial infection [48]. Inhibition of PLA_2 is a common pathogenic mechanism of these entomopathogenic bacteria [32]. In an active way, the symbiotic bacteria, including *X. nematophila*, adhere to and kill the hemocytes through their lipopolysaccharides or by release of some cytotoxic factors [9, 16, 43]. Cho and Kim [10] showed that the hemocytes infected with *X. nematophila* followed a typical apoptotic process. From the culture broth of *X. nematophila*, three phenethylamide compounds were purified and shown for the presence of potent cytotoxic substances [39], where N-phenethyl-2-phenylacetamide caused apoptosis of human tumor cells by activating cytochrome *c*-dependent caspase-3 [20]. At this moment, we do not know how *P. vermicola* and *Flavobacterium* sp. induce their potent entomopathogenic capacity, which has to be further investigated to better understand the pathogenic interactions played between these two bacteria and the host nematode.

Acknowledgments

This study was funded by the 2006 Special Grant of Andong National University to YL. SS and JS were supported by the Second Stage BK21 program of the Ministry of Education & Human Resources Development, Korea. Yi, Young-Keun and Hae Woong Park contributed equally to this work.

References

1. Adams, B. J., A. M. Burnell, and T. O. Powers. 1998. A phylogenetic analysis of Heterorhabditis (Nemata: Rhabditidae) based on internal transcribed spacer 1 DNA sequence data. *J. Nematol.* **30:** 22–39.
2. Aguillera, M. and G. Smart. 1993. Development, reproduction and pathogenicity of *Steinernema scapterisci* in monoxenic culture with different species of bacteria. *J. Invertebr. Pathol.* **62:** 289–294.
3. Akhurst, R. J. 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *J. Gen. Microbiol.* **121:** 303–309.
4. Benson, H. G. 1990. *Microbiological Applications*, 4th Ed. Wm. C. Brown Publishers, Dubuque, IA.
5. Blaxter, M. L., P. De Ley, J. R. Garey, L. X. Liu, P. Scheldeman, A. Vierstraete, J. R. Vanflateran, L. Y. Mackey, M. Dorris, L. M. Frisse, J. T. Vida, and W. K. Thomas. 1998. A molecular framework for the phylum Nematoda. *Nature* **392:** 71–75.
6. Boemare, N. E. 2002. Biology, taxonomy and systematics of photorhabdus and xenorhabdus, pp. 35–56. *In* R. Gaugler (ed.). *Entomopathogenic Nematology.* CABI Publishing, New York, NY.
7. Brenner, D. J. 1984. Family I. *Enterobacteriaceae*, pp. 408–516. *In* N. R. Krieg and J. G. Holt (eds.). *Bergey's Manual of Systematic Bacteriology*, Vol. 1. Williams & Wilkins, Baltimore, MD.
8. Brewer, M. J. and J. T. Trumble. 1989. Field monitoring for insecticide resistance in beet armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* **82:** 1520–1526.
9. Brillard, J., C. Ribeiro, N. Boemare, M. Brehelin, and A. Givaudan. 2001. Two distinct hemolytic activities in *Xenorhabdus nematophila* are active against immunocompetent insect cells. *Appl. Environ. Microbiol.* **67:** 2515–2525.
10. Cho, S. and Y. Kim. 2004. Hemocyte apoptosis induced by entomopathogenic bacteria, *Xenorhabdus* and *Photorhabdus*, in *Bombyx mori*. *J. Asia-Pacific Entomol.* **7:** 195–200.
11. Cho, S. J., K. M. Cho, E. C. Shin, W. J. Lim, S. Y. Hong, B. R. Choi, J. M. Kang, S. M. Lee, Y. H. Kim, H. Kim, and H. D. Yun. 2006. 16S rDNA analysis of bacterial diversity in three fractions of cow rumen. *J. Microbiol. Biotechnol.* **16:** 92–101.
12. Converse, V. and P. S. Grewal. 1998. Virulence of entomopathogenic nematodes to the Western masked chafer *Cyclocephala hirta* (Coleoptera: Scarabaeidae). *J. Econ. Entomol.* **91:** 428–432.

13. Dowds, B. C. A. and A. Peters. 2002. Virulence mechanisms, pp. 79–98. *In* R. Gaugler (ed.). *Entomopathogenic Nematology*. CABI Publishing, New York.
14. Dunphy, G. B. and J. M. Webster. 1984. Interaction of *Xenorhabdus nematophilus* subsp. *nematophilus* with the haemolymph of *Galleria mellonella*. *J. Insect Physiol.* **30:** 883–889.
15. Dunphy, G. B. and J. M. Webster. 1987. Partially characterized components of the epicuticle of dauer juvenile, *Steinernema feltiae*, and their influence on haemocyte activity in *Galleria mellonella*. *J. Parasitol.* **73:** 584–588.
16. Dunphy, G. B. and J. M. Webster. 1991. Antihemocytic surface components of *Xenorhabdus nematophilus* var. *dutki* and their modification by serum of nonimmune larvae of *Galleria mellonella*. *J. Invertebr. Pathol.* **58:** 40–51.
17. Forst, S., B. Dowds, N. Boemare, and E. Stackebrandt. 1997. *Xenorhabdus* and *Photorhabdus* spp.: Bugs that kill bugs. *Annu. Rev. Microbiol.* **51:** 47–72.
18. Gho, H. G., S. G. Lee, B. P. Lee, K. M. Choi, and J. H. Kim. 1990. Simple mass-rearing of beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), on an artificial diet. *Kor. J. Appl. Entomol.* **29:** 180–183.
19. Grewal, P. S., E. E. Lewis, R. Gaugler, and J. F. Campbell. 1994. Host-finding behaviour as a predictor of foraging strategy in entomopathogenic nematodes. *Parasitology* **108:** 207–215.
20. Hwang, S. Y., S. Paik, S. H. Park, H. S. Kim, I. S. Lee, S. P. Kim, W. K. Baek, M. H. Suh, T. K. Kwon, J. W. Park, J. B. Park, J. J. Lee, and S. I. Suh. 2003. N-Phenethyl-2-phenylacetamide isolated from *Xenorhabdus nematophilus* induced apoptosis through caspases activation and calpain-mediated Bax cleavage in U937 cells. *Int. J. Oncol.* **22:** 151–157.
21. Holmes, B., R. J. Owen, and T. A. McMeekin. 1984. Genus *Flavobacterium*, pp. 353–361. *In* N. R. Krieg and J. G. Holt (eds.). *Bergey's Manual of Systematic Bacteriology*, Vol. 1. Williams & Wilkins, Baltimore, MD.
22. Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams 1994. *Bergey's Manual of Determinative Bacteriology*, 9th Ed. Williams & Wilkins, Baltimore, MD.
23. Humason, G. L. 1972. *Animal Tissue Techniques*, 3rd Ed. W. H. Freeman and Company, San Francisco, CA.
24. Hunt, D. J. 1980. *Butlerius macrospiculum* n. sp. and *Cylindrocorpus walkeri* n. sp. (Nematoda: Diplogastroidea) from St. Lucia, West Indies. *Revue Nématol.* **3:** 155–160.
25. Ji, D., Y. Yi, G. H. Kang, Y. H. Choi, P. Kim, N. I. Baek, and Y. Kim. 2004. Identification of an antibacterial compound, benzylideneacetone, from *Xenorhabdus nematophila* against major plant-pathogenic bacteria. *FEMS Microbiol. Lett.* **239:** 241–248.
26. Jung, S. and Y. Kim. 2006. Synergistic effect of *Xenorhabdus nematophila* K1 and *Bacillus thuringiensis* subsp. *aizawai* against *Spodoptera exigua* (Lepidoptera: Noctuidae). *Biol. Control* **39:** 201–209.
27. Jung, S. and Y. Kim. 2006. Synergistic effect of entomopathogenic bacteria (*Xenorhabdus* sp. and *Photorhabdus temperata* ssp. *temperata*) on the pathogenicity of *Bacillus thuringiensis* ssp. *aizawai* against *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environ. Entomol.* **35:** 1584–1589.
28. Jung, S. and Y. Kim. 2007. Potentiating effect of *Bacillus thuringiensis* subsp. *kurstaki* on pathogenicity of entomopathogenic bacterium *Xenorhabdus nematophila* K1 against diamondback moth (Lepidoptera: Plutellae). *J. Econ. Entomol.* **100:** 246–250.
29. Kaya, H. K. 1990. Soil ecology, pp. 215–231. *In* R. Gaugler and H. K. Kaya (eds.), *Entomopathogenic Nematodes in Biological Control*. CRC, Boca Raton, FL.
30. Kaya, H. K. and R. Gaugler. 1993. Entomopathogenic nematodes. *Annu. Rev. Entomol.* **38:** 181–206.
31. Kim, J. S., J. Y. Choi, J. H. Chang, H. J. Shim, J. Y. Rho, B. R. Jin, and Y. H. Je. 2005. Characterization of an improved recombinant baculovirus producing polyhedra that contain *Bacillus thuringiensis* Cry1Ac crystal protein. *J. Microbiol. Biotechnol.* **15:** 710–715.
32. Kim, Y., D. Ji, S. Cho, and Y. Park. 2005. Two groups of entomopathogenic bacteria, *Photorhabdus* and *Xenorhabdus*, share an inhibitory action against phospholipase A_2 to induce host immunodepression. *J. Insect Physiol.* **89:** 258–264.
33. Lee, S., Y. Kim, and S. Han. 2000. An improved collecting method of the infective juveniles of the entomopathogenic nematode, *Steinernema carpocapsae* Weiser. *Kor. J. Soil Zool.* **5:** 97–100.
34. Lee, Y. O., J. H. Park, and J. K. Park. 2005. Microbial characterization of excessive growing biofilm in sewer lines using molecular technique. *J. Microbiol. Biotechnol.* **15:** 938–945.
35. Mahar, A. N., M. Munir, S. Elawad, S. R. Gowen, and N. G. M. Hague. 2004. Microbial control of diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) using bacteria (*Xenorhabdus nematophila*) and its metabolites from the entomopathogenic nematode *Steinernema carpocapsae*. *J. Zhejiang Univ. Sci.* **5:** 1183–1190.
36. Martens, E. C. and H. Goodrich-Blair. 2005. The *Steinernema carpocapsae* intestinal vesicle contains a subcellular structure with which *Xenorhabdus nematophila* associates during colonization initiation. *Cell Microbiol.* **7:** 1723–1735.
37. Martens, E. C., K. Heungens, and H. Goodrich-Blair. 2003. Early colonization events in the mutualistic association between *Steinernema carpocapsae* nematodes and *Xenorhabdus nematophila* bacteria. *J. Bacteriol.* **185:** 3147–3154.
38. Navon, A., S. Keren, L. Salame, and I. Glazer. 1998. An edible-to-insects calcium alginate gel as a carrier for entomopathogenic nematodes. *Biocon. Sci. Tech.* **8:** 429–437.
39. Paik, S., Y. H. Park, S. I. Suh, H. S. Kim, I. S. Lee, M. K. Park, C. S. Lee, and S. H. Park. 2001. Unusual cytotoxic phenethylamides from *Xenorhabdus nematophilus*. *Bull. Korean Chem. Soc.* **22:** 372–374.
40. Park, Y. and Y. Kim. 2000. Eicosanoids rescue *Spodoptera exigua* infected with *Xenorhabdus nematophilus*, the symbiotic bacteria to the entomopathogenic nematode *Steinernema carpocapsae*. *J. Insect Physiol.* **11:** 1469–1476.
41. Penner, J. L. 1984. Genus XII. *Providencia*, pp. 494-496, *In* N. R. Krieg and J. G. Holt (eds.). *Bergey's Manual of*

Systematic Bacteriology, Vol. 1. Williams & Wilkins, Baltimore, MD.
42. Raymond, M. 1985. Presentation d'un programme d'analyse log-probit pour micro-ordinateur. Cah. ORSTOM. *Ser. Ent. Med. Parasitol.* **22:** 117–121.
43. Ribeiro, C., B. Duvic, P. Oliveira, A. Givaudan, F. Palha, N. Simoes, and M. Brehelin. 1999. Insect immunity - effects of factors produced by a nematobacterial complex on immunocompetent cells. *J. Insect Physiol.* **45:** 677–685.
44. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning, A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
45. Schaad, N. M. 1988. *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, 2nd Ed. APS Press, St. Paul, MN.
46. Somvanshi, V. S., E. Lang, B. Straubler, C. Sproer, P. Schumann, S. Ganguly, A. K. Saxena, and E. Stackebrandt. 2006. *Providencia vermicola* sp. nov., isolated from infective juveniles of the entomopathogenic nematode *Steinernema thermophilum*. *Int. J. Syst. Evol. Microbiol.* **56:** 629–633.
47. Somvanshi, V. S., E. Lang, S. Ganguly, J. Swiderski, A. K. Saxena, and E. Stackebrandt. 2006. A novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus indica* sp. nov., symbiotically associated with entomopathogenic nematode *Steinernema thermophilum* Ganguly and Singh, 2000. *Syst. Appl. Microbiol.* **56:** 629–633.
48. Stanley, D. W. 2000. *Eicosanoids in Invertebrate Signal Transduction Systems*. Princeton University Press, Princeton, NJ.
49. Stock, S. P., J. F. Campbell, and S. A. Nadler. 2001. Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *J. Parasitol.* **87:** 877–889.
50. Van Laecke, K. and D. Degheele. 1991. Synergism of diflubenzuron and teflubenzuron in larvae of beet armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* **84:** 785–789.
51. Vrain, T. C., D. A. Wakarchuk, A. C. Levesque, and R. I. Hamilton. 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fund. Appl. Nematol.* **15:** 563–574.
52. Walsh, K. T. and J. M. Webster. 2003. Interaction of microbial populations in *Steinernema* (Steinernematidae, Nematoda) infected *Galleria mellonella* larvae. *J. Invertebr. Pathol.* **83:** 118–126.
53. Wang, Y., J. F. Campbell, and R. Gaugler. 1995. Infection of entomopathogenic nematodes *Steinernema glaseri* and *Heterorhabditis bacteriophora* against *Popillia japonica* (Coleoptera: Scarabaeidae) larvae. *J. Invertebr. Pathol.* **66:** 178–184.
54. Webster, J. M., G. Chen, and J. Li. 1998. Parasitic worms: An ally in the wax against the superbugs. *Parasitol. Today* **14:** 161–163.
55. Weisburg, G. W., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173:** 697–703.