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Isolation and Identification of a Symbiotic Bacterium from *Steinernema carpocapsae*

Sun Ho Park* **and Yeon Su Yu**

School of Chemical Engineering & Materials Engineering, Keimyung University, Taegu 704-701, Korea

Xenorhabdus nematophilus sp., an insect-pathogenic bacterium, was newly isolated from Korean entomopathogenic nematode of *Steinernema carpocapsae,* which can be used as a useful bioinsecticide. Primary and secondary form variants of *Xenorhabdus nematophilus* were observed when cultured *in vitro.* Primary form variants adsorbed bromothymol blue, while secondary form did not. However, many other characters of two variants were very similar. The variants were all rod-shaped and cell size was highly variable ranging from 0.5 by 2.0 μm to 1.0 by 5.0 μm. Both produced highly toxic substances and killed the insect larva within 20~38 hr, indicating that insect pathogenicity of *Xenorhabdus* is not directly associated with its phase variation. In addition, cell-free culture supernatant of *Xenorhabdus* was sufficient to kill the insect larva by injecting it into insect hemolymph; however, cell-harboring culture broth was more effective for killing the insect. The use of *Xenorhabdus nematophilus* may provide a potential alternative to *Bacillus thuringiensis* (Bt) toxins.

Key words: *Steinernema carpocapsae*, entomopathogenic nematode, *Xenorhabdus nematophilus*, symbiotic bacterium, biological insecticide

INTRODUCTION

Recently, interest of biological control was remarkably increased because of environmental damage resulting from chemical pesticides. Entomopathogenic nematodes have been successfully used in the biological control of pest insects and are safe for non-target organisms [1,2]. So far nearly 40 nematode families are reported to be associated with insects, but very few of these nematodes have the ability to search for and kill hosts rapidly. At present, nematode families of *Steinernematidae* and *Heterorhabditidae* are widely available for use in biological pesticides which can serve as alternatives to chemical pesticides [3].

Xenorhabdus and *Photorhabdus* spp. are two genera of bacteria in the intestines of the third stage infective juveniles (IJs) of nematodes belonging to the families *Steinernematidae* and *Heterorhabditidae*, respectively [4]. The IJs are able to invade and kill the larval stage of numerous insects. And this is very ideal system to control insect pest, because unlike most other systems of biological control (e.g., *Bacillus thuringiensis*), the nematode actively seeks out prey larvae. Both *Xenorhabdus* and *Photorhabdus* spp. are motile Gram negative, facultatively anaerobic, rod shaped bacteria currently assigned to the family Enterobacteriaceae [5]. The nematodes enter through mouth, anus or spiracles at larval stage of different kinds of insects and subsequently penetrate into the hemolymph of the host insect. Upon entrance into the hemolymph, the nematodes release the bacteria into the hemolymph. Both the nematodes and the bacteria rapidly kill the insect larvae. As the bacteria enter the stationary phase of their growth cycle within the hemolymph of the larval carcass, they secrete several extracellular products, including lipases, phospholipases, proteases, and several different broad-spectrum antibiotics. The degradative enzymes break down the macromolecules of the insect cadaver to provide the developing nematode with a nutrient supply, while the antibiotics suppress contamination of the cadaver by other microorganisms [6-8]. Another intriguing property of *Xenorhabdus* and *Photorhabdus* spp. is the formation of phenotypic variant forms that can be isolated at low and variable frequencies during prolonged incubation under stationary conditions. The variant form's, or so-called phase II cells, are altered in many properties and are not found as natural symbionts in the nematode. Phase I cells are the form of the bacteria that naturally associate with the infective juvenile nematode [9]. Although the isolation of symbiotic bacteria has been reported in many countries, no systematic survey has been conducted for Korean entomopathogenic nematodes. Very recently, *Xenorhabdus nematophilus* has been isolated from *Steinernema glaseri* Dongrae which was collected at Pusan, Korea [10].

In this work, we have isolated and identified a symbiotic bacterium from *Steinernema carpocapsae* nematode which was collected at Pocheon located in the middle of the Korean Peninsula. Insect pathogenicity and culture characteristics of the isolates were also analyzed to examine more closely the significance of entomopathogenic nematode associated with its symbiotic bacteria.

* Corresponding author
Tel : +82-53-580-5457 Fax : +82-53-633-4929
e-mail : park@kmucc.keimyung.ac.kr

MATERIALS AND METHODS

Materials

MacConkey agar, nutrient agar (NA), yeast extract, phenolphthalein diphosphate, peptone from soymeal, Tween 85, L-phenylalanine, ferric chloride were purchased from Merck. Bromothymol blue was from Junsei, triphenyltetrazolium chloride was from Sigma. Biolog Universal Growth Medium (BUGM) and microplate coated with 95 carbon sources were from Biolog (Biolog, U.S.A.).

Organism and Growth Conditions

Infective juvenile (IJ) nematodes of *Steinernema carpocapsae* Pocheon, originally collected by Kyongsang National University (Chinju, Korea), were used in this study [11]. Surface-sterilized IJs harboring symbiotic bacteria were incubated in YS medium (0.05% $NH_4H_2PO_4$ + 0.05% K_2HPO_4 + 0.02% $MgSO_4 \cdot 7H_2O$ + 0.5% NaCl + 0.5% yeast extract) at 28℃. The bacteria isolates from *Steinernema carpocapsae* Pocheon were subcultured on nutrient agar (NA) or NBTA (NA + 0.0025% bromothymol blue + 0.004% triphenyltetrazolium chloride) at 25℃. Larvae of the greater wax moth *Galleria mellonella* were reared to the third instar by the method of Park and Yu [12]. Larvae weighing 180±3 mg each were used in the experiments.

Isolation and Identification of Symbiotic Bacteria

Steinernema carpocapsae nematodes stored at 10℃ were warmed at 25℃ for 30 min until its activity was recovered. When any contamination was found using microscope, IJs were treated 2 or 3 times with 0.1% formalin and rinsed 3 times with sterile distilled water (sdw). IJs were then sedimented in a 100 mL beaker for 30 min and supernatant was decanted. To sterilize surface of IJs, they were soaked in 0.5% NaClO solution for 10 min and rinsed 2 times with sdw. The following three methods were employed to isolate symbiotic bacteria from surface-sterilized IJs. First, surface-sterilized IJs were destroyed by vortexing and transferred to incubated to a 250 mL flask containing YS medium and cultured at 28℃ and 200 rpm for 48 hr in a shaking incubator. Culture broth was then spreaded onto MacConkey agar or NBTA and incubated at 25℃ for 48 hr. Second, a hemolymph of surface-sterilized *Galleria mellonella* larva was aseptically collected into NA plate and surface-sterilized IJs were inoculated. After 48 hr of incubation at 25℃, strains grown were transferred into NBTA and incubated at 25℃ for 48 hr. Third, *Galleria mellonella* larvae were infected with IJs, incubated at 25℃ for 48 hr and sterilized with 95% ethanol. Larvae were carefully opened with sterile forceps, infected hemolymphs were collected into NBTA and incubated at 25℃ for 48 hr.

For indentification of bacteria isolates colony on NA plate was streaked onto BUGM. After 3 days of cultivation, the strains were diluted with 0.85% saline solution to adjust cell concentration appropriately. The strains were loaded into 96 well microplate used for Biolog system. After 24 hr of incubation at 25℃, microplate was inserted into the Biolog identification system. Oxidation quotients were estimated for each of 95 carbon sources and the data were analyzed via 556 species/genus Gram negative strains database [10,12].

Physiological and Biochemical Characterization of Symbiotic Bacteria

Physiological and biochemical characters of symbiotic bacteria were examined by the procedures described elsewhere [13]. To distinguish primary and secondary forms of symbiotic bacteria, NBTA test based on adsorption of bromothymol blue was conducted [14]. Biochemical tests for catalase, lecithinase, urease, phosphatase and lipolysis (Tween 85) were conducted to distinguish *Xenorhabdus* spp. as described by Akhurst [14,15].

Insect Pathogenicity of Symbiotic Bacteria

Insect pathogenicity of symbiotic bacteria was examined by using *Galleria mellonella* larvae. The colony on NBTA plate was suspended with saline solution and diluted into various concentrations. A 5 μL of cell suspension was injected into the hemolymph of larva with microsyringe. To examine whether cells secrete toxic substances extracellularly, bacteria were also cultured at 28℃ for 48 hr on YS medium. Culture broth was centrifuged at 10,000 rpm for 15 min, and culture supernatant was injected into the hemolymph of *Galleria mellonella* larva. For each sample, five larvae of *Galleria mellonella* were used and incubated at 25℃. The mean lethal times were estimated by the method described earlier [16].

Electron Microscopy

The bacterium was suspended in 0.9% NaCl solution and 1 or 2 drops of cell suspension was applied to the Whatman paper. After the cells were grown at 28℃ for 2 days, the cells were fixed with 2.5% glutaraldehyde in 0.2 M PBS, pH 7.3 and they were post-fixed with 1% OsO_4 in PBS at 4℃ for 2 hr. The samples were washed 3 times with 0.2 M phosphate buffer (pH 7.3), dehydrated a graded series of ethanol, and immersed in isoamylacetate overnight. These specimens were dried in a critical point dryer and then coated with gold in an ion coater. They were viewed with a scanning electron microscope (Hitachi S-4100, Japan).

RESULTS AND DISCUSSION

Isolation and Characterization of Symbiotic Bacteria

With the exception of infected host, the isolation methods resulted in only one colony of symbiotic bacterium (XR-PC). From the hemolymph of infected hosts, four types of bacteria were isolated and XR-PC was reisolated in NBTA medium. The other three bacteria isolates seem to come from gut of *Galleria mellonella* larva. One of them was identified as *Xanthomonas maltophilia* which is an important cause of nosocomial infections, especially in immunocompromised patients [12].

Major characteristics of bacteria isolates (XR-PC) from *Steinernema carpocapsae* are presented in Table

Table 1. Characteristics of symbiotic bateria isolates with phase variation

Characteristics	Phase I	Phase II
Pigment	yellow	yellow
Bioluminescence	-	-
Insect pathogenicity	+	+
Growth on 37℃	-	-
Colony colour on MacConkey agar	red-brown	bright pink
Adsorption of bromothymol blue	+	-
Catalase	-	-
Lecithinase	+	+
Urease	-	-
Phosphatase	-	-
Lipolysis (Tween 85)	+	+

1. We have recently reported the symbiotic bacterium (XR-DR), *Xenorhabdus nematophilus*, isolated from *Steinernema glaseri* nematode [10]. Despite the fact that both XR-PC and XR-DR were isolated from *Steinernema* genera, remarkable differences in physiological and biochemical characters were found between them. XR-DR did not hydrolyse Tween 85, but produced phosphatase. In contrast, the results of XR-PC were vice versa. XR-PC did not grow at 37℃, while XR-DR grew at 37℃. In phenotypic variations, XR-PC produced two colony types on agar media and showed very similar characters to XR-DR. The primary form (phase I) was unstable *in vitro*, and produced secondary form (phase II). In colony color, both phase I and II were yellow in NA, but the phase I differed from the phase II in adsorption of dyes from agar media. As shown in Fig. 1, phase I was blue or green but phase II was red in the NBTA. Phase I colony was surrounded by cleared zones in the MacConkey and NBTA because neutral red and BTB has been adsorbed, respectively. Phase I was mucoid and very difficult to disperse in water but phase II was easily dispersed. However, two forms of XR-PC gave very similar responses to many other biochemical and physiological tests.

The occurrence of two forms in symbiotic bacteria associated with entomopathogenic nematodes has been known to be a characteristics of the genus *Xenorhabdus* [14,15]. In general, phase I has superior ability to provide nutrients for the nematodes in insects and artificial media. Phase I characters include binding dyes and producing antibiotic metabolites, lecithinase, proteinaceous inclusion bodies and fimbriae; Phase II either does not have these characters or expresses them weakly [6]. Phase change in *Xenorhabdus* spp. very rarely occurs *in vivo* but readily *in vitro*. It occurs during the stationary phase of the culture cycle and its extent and timing are highly variable. The exact mechanism of phase variation in *Xenorhabdus* is not known, but it differs from the general rule in that it involves a reversible change related to host's immune defences or bacteriophage infection [13]. Genetic mechanisms which regulate the switch from phase I to phase II should be identified in order to elucidate an integral part of the interaction between nematodes and their symbionts.

Identification of Symbiotic Bacteria

Fig. 2 shows morphological character of rod-shaped XR-PC. Cell size was highly variable ranging from 0.5 by 2.0 μm to 1.0 by 5.0 μm and occasionally with filaments 15~50 μm in length. But it was difficult to distinguish morphological characters of phase I and II form.

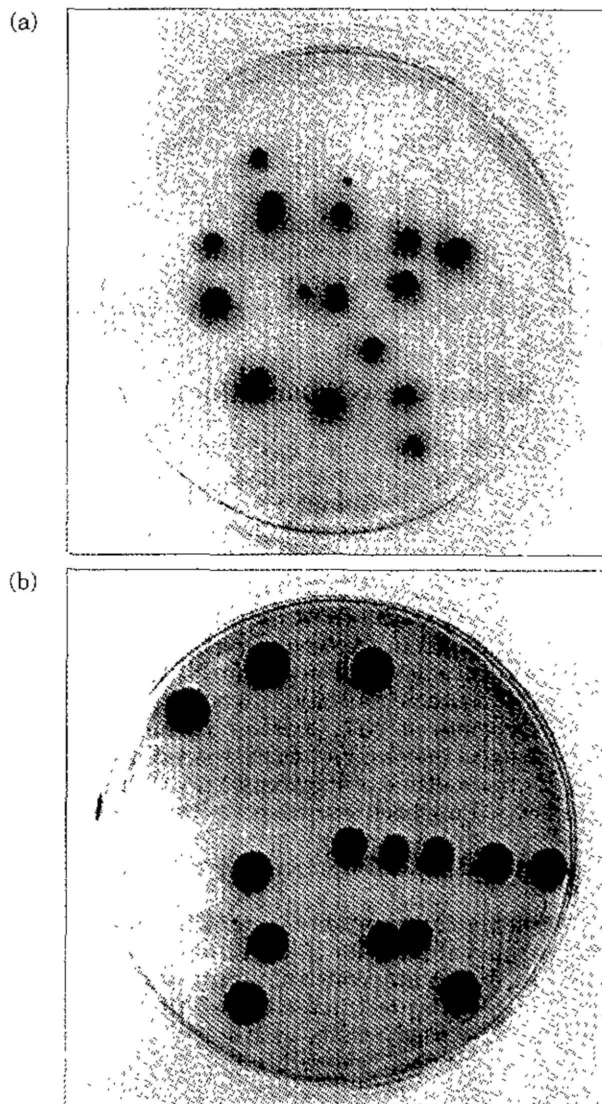

Fig. 1. Adsorption of dyes from NBTA in distinguishing phase I and II of bateria isolate: (a) Phase I, (b) Phase II.

The utilization of carbon sources by XR-PC was analyzed and compared with 566 species/genus Gram negative strains in identification system. Recently, Zak *et al.* [17] used Biolog identification system to separate microorganism living in the soil of desert. Biolog identification system was a useful method because each microorganism had specific degree and rate of oxidation on each substrate. The 95 assimilation characters for XR-PC provided useful data for the analyses and are listed in Table 2. When assimilation data of XR-PC were compared with those of XR-DR [10], Tween 40 and L-proline showed opposite responses between two strains, as positive in one strain and as negative in a second. Moreover, there were some slight differences in results for assimilation of other 20 carbon sources including D-fructose, m-inositol and D-gluconic acid. Generally, correct identification of an unknown strain requires the similarity over 0.5 unless other strains were found. In this study, the identification result of XR-PC was obtained as *Xenorhabdus nematophilus* subsp. *bovienii* with the similarity 0.533 and named as *Xenorhabdus nematophilus* PC1.

Fig. 2. Scanning electron micrograph of bacteria isolated from *Steinernema carpocapsae*.

Insect Pathogenicity of Symbiotic Bacteria

Fig. 3 shows mean lethal times of *Galleria mellonella* larvae at various concentrations of bacteria isolates. Injected number of bacterial isolates per larva were varied from 0 to 2×10^5. Injection of 60~80 cells of the bacteria per larva was sufficient to kill the insect larva within 40 hr. Insect mortality occurred before significant numbers of bacteria were present in the hemolymph of insect larva(data not shown). This implies that *Xenorhabdus nematophilus* produces insect pathogens which act early in the infectious process. The mean lethal time was decreased to 20 hr as the bacterium concentration was increased up to $6\sim8\times10^3$ cells/larva. Meanwhile, both phase I and II strains were all effective for killing *G. mellonella*, indicating that insect pathogenicity of *Xenorhabdus nematophilus* is not directly associated with its phase variation (Table 1). When cell-free culture supernatant was injected into hemolymph, it also killed the insect larva within 30 hr. This indicates that the bacterium produces major toxic factors extracellularly. Like many other Gram negative bacteria, *Xenorhabdus nematophilus* can produce endotoxins. One of *Xenorhabdus nematophilus* endotoxins are known as lipopolysaccharide components of the cell wall [18]. However, it is not clear that the endotoxin alone is sufficient to kill the host. A variety of insecticidal exotoxins such as proteases, phospholipases and lipases have been also reported in other bacteria [13]. These enzymes have not yet been proved to be the active agents in *Xenorhabdus* toxicity for insects. Recently, Bowen *et al.* [19] purified high molecular weight protein with high oral toxicity to *Manduca sexta* larvae from *Photorhabdus luminescens*. The data presented in this study suggest that the entomopathogenecity of *Steinernema carpocapsae* are mainly attributable to extracellular toxic factors associated with *Xenorhabdus nematophilus*. Even though the toxic substances have not been identified yet, they were apparently as potent as the endotoxin of *Bacillus thuringiensis*(data not shown). Further studies on purification and characterization of these substances are currently under investigation.

Table 2. Assimilation of carbon source for bacteria isolated from entomopathogenic nematode *Steinernema carpocapsae*

Carbon	XR-PC(I)	Carbon	XR-PC(I)	Carbon	XR-PC(I)
water	-	turanose	-	D-alanine	w
α-cyclodextrin	-	xylitol	-	L-alanine	+
dextrin	+	methyl pyruvate	+	L-alanyl-glycine	w
glycogen	-	mono-methyl succinate	-	L-asparagine	+
Tween 40	-	acetic acid	-	L-aspartic acid	+
Tween 80	w	cis-aconitic acid	-	L-glutamic acid	+
N-acetyl-D-galactosamine	-	citric acid	-	glycyl-L-aspartic acid	-
N-acetyl-D-glucosamine	+	formic acid	w	glycyl-L-glutamic acid	+
adonitol	-	D-galactonic acid latone	-	L-histidine	w
L-arabinose	w	D-galacturonic acid	-	hydroxy L-proline	-
D-arabitol	-	D-gluconic acid	-	L-leucine	-
cellobiose	-	D-glucosaminic acid	-	L-omithine	-
i-erythritol	-	D-glucuronic acid	-	L-phenylalanine	-
D-fructose	+	α-hydroxybutyric acid	-	L-proline	+
L-fucose	-	β-hydroxybutyric acid	-	L-pyroglutamic acid	-
D-galactose	-	γ-hydroxybutyric acid	-	D-serine	w
gentiobiose	-	p-hydroxyphenylacetic acid	+	L-serine	+
α-D-glucose	+	itaconic acid	-	L-threonine	-
m-inositol	w	α-keto butyric acid	-	D,L-carnitine	-
α-D-lactose	-	α-keto glutaric acid	-	γ-amino butyric acid	-
lactulose	-	α-keto valeric acid	-	urocanic acid	-
maltose	+	D,L-lactic acid	w	inosine	+
D-mannitol	-	malonic acid	-	uridine	+
D-mannose	+	propionic acid	w	thymidine	w
D-melibiose	-	quinic acid	-	phenyl ethylamine	w
β-methyl-D-glucoside	-	D-saccharic acid	-	putrescine	w
D-psicose	-	sebacin acid	-	2-amino ethanol	-
D-raffinose	-	succinic acid	w	2,3-butanediol	-
L-rhamnose	-	bromosuccinic acid	-	glycerol	+
D-sorbitol	-	succinamic acid	-	D,L- α-glycerol phosphate	+
sucrose	-	glucuronamide	w	glucose-1- phosphate	+
D-trehalose	+	alaninamide	w	glucose-6- phosphate	+

+, positive response; -, negative response; w, weak response

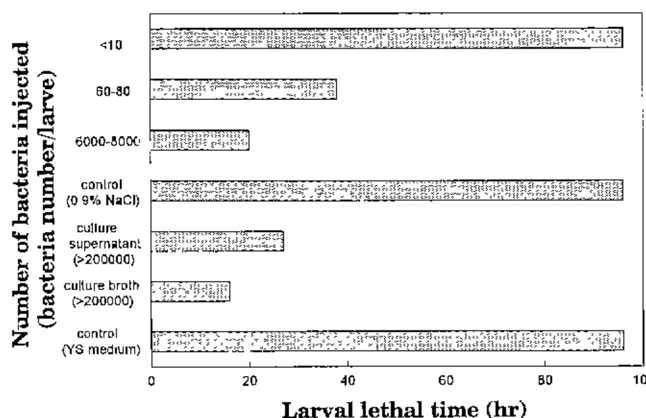

Fig. 3. Pathogenicity of *Xenorhabdus nematophilus* against *Galleria mellonella* larva.

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