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Insecticidal Activity and Genetic Characterization of Four Bacterial Isolates of *Xenorhabdus* and *Photorhabdus* Associated with Entomopathogenic Nematodes

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ABSTRACT

Three *Photorhabdus luminescens* and one *Xenorhabdus nematophila* strains were isolated from *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* nematodes, respectively. The nematodes were isolated from soil samples collected from four Egyptian governorates using *Galleria* larvae as bait. Toxicity of the four isolates on the 3rd instar wax moth larvae of *Galleria mellonella* L. showed that BA1 and MH had the highest mortality (66.6 and 60%, respectively) at a concentration of 5.35×10^3 after 9 days using direct feeding. By using direct spray, BA1 displayed the highest mortality (73.3%) followed by MH (66.6%) and BAA1 (60%) after 8 days at the same concentration. Using direct injection, BA1 and BA2 revealed complete mortality after 48 h and after 24 h BA1 showed 90 and 70% mortality with 0.67×10^3 and 0.33×10^3 concentrations, respectively followed by BA2 with 80 and 70% mortality with 1.04×10^3 and 0.52×10^3 concentrations, respectively. The other two isolates showed complete mortality after 72 h. Moreover, at 2.67×10^3 BAA1 showed a high mortality (60%) after 8 days of direct spray. BA2 displayed a similar high mortality after 120 h of direct feeding and spray with 60% using three concentrations (8.35×10^3, 2.08×10^3 and 1.04×10^3). RAPD analysis revealed 46 polymorphic fragments that ranged in size from 7.25 to 0.15 kb. The mean percentage of polymorphism shown by the four primers was 67.6%, decreasing in this order: UBC-37 (75%), UBC-89 (73.3%), UBC-16 (65%) and UBC-28 (58.8%). BA2, belonging to *Xenorhabdus nematophila*, had the highest number (6) of specific fragments, followed by MH with two and both BA1 and BAA1 with one. SDS-PAGE of proteins displayed remarkable genetic variation between the four isolates. Several toxic protein bands were detected among the four isolates, for instance 40 kD in BA2, 283 kD in BA1, BAA1 and MH and 37 kD in BA1 and MH.

Keywords: *Galleria mellonella*, larvae, local *Xenorhabdus* and *Photorhabdus* isolates, RAPD-PCR, SDS-PAGE proteins

INTRODUCTION

Steinernematidae and Heterorhabditidae are two families of entomopathogenic nematodes which are strongly virulent against a wide range of insects. Gaugler and Kaya (1990) suggested that they could be raised for use in biological control of insect pests. All *Steinernema* spp. carry in their gut symbiotic bacteria of the genus *Xenorhabdus*, and all *Heterorhabditis* spp. carry symbiotic bacteria of the genus *Photorhabdus* (Fischer-Le Saux *et al*. 1998). There are no reports of the isolation of *Xenorhabdus* and *Photorhabdus* from soil and it has been generally assumed that these bacteria cannot exist in the soil environment in the absence of their nematode associates (Felföldi *et al.* 2007). With emerging resistance to *Bacillus thuringiensis* (*Bt*) among insects, *Xenorhabdus* and *Photorhabdus* are considered the next generation of microbial insecticides. They also known to produce insecticidal toxins and are closely related to *E. coli*. However, unlike *Bt*, *Xenorhabdus* and *Photorhabdus* toxins are not toxic when ingested by the insect. The symbiotic association plays an important role in both reproduction and pathogenicity of the nematodes (Boemare *et al*. 1996, 1997). After penetration into the body cavity of the insect, the nematodes release their symbiotic bacteria into the insect hemolymph, inducing death by toxaemia and septicaemia (Peel *et al.* 1999). Entomopathogenic nematodes (EPN) of the family Steinernematidae, which belong to important parasites of many insect species, are successfully used in biological control of many pest insects instead of using the conventional chemical pest control methods that are recently criticized for their adverse environmental effects (Shapiro-Ilan *et al.* 2002).

Xenorhabdus and *Photorhabdus* spp. appear to display a high and monophyletic diversity: five *Xenorhabdus* species (*X. nematophia*, *X. poinarii*, *X. bovienii*, *X. beddingii* and *X. japonicas*), and only one *Photorhabdus luminescens* species were previously described (Boemare *et al.* 1993). However, within *P. luminescens* several genomic groups have been recognized by DNA-DNA hybridization and suggested by 16S rDNA sequencing. In both genera, identification of new bacterial isolates is difficult because most strains are phenotypically very similar and fail to give positive results in many classical tests for identification (Szállás *et al.* 1997). Consequently, only a few species have been described, and some of these are represented by only a few isolates. Ecological data relating bacterial symbionts with their nematode host or their environment remain weak. Thus, studies on the taxonomic diversity and distribution of members of *Xenorhabdus* and *Photorhabdus* spp. are needed. Isolation and identification of native nematode-bacterial associations in the field are necessary for successful control of endemic pests in a particular location. Besides, an increase of local strains would be more suitable to control known pests in this area and would be well adapted to the Egyptian environment. Molecular methods can be employed to determine diversity among bacteria or used for rapid identification of a bacterium in question so as to avoid laborious phenotypic characterization (Adams *et al.* 2006). Genotypic diversity of some bacterial species and isolates is being increasingly analyzed by RAPD-PCR and SDS-PAGE

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Table 1 *Photorhabdus luminescens* and *Xenorhabdus nematophila* strains, their nematode host species and governorate origins.

Strain	Bacteria	Nematodes	Origin area and governorates
BA2	*Xenorhabdus nematophila*	*Steinernema carpocapsae*	**El-Arish city (North Sinai)
BA1	*Photorhabdus luminescens*	*Heterorhabditis bacteriophora*	**Kefor El-Nile area (Fayoum)
BAA1	*P. luminescens*	*H. bacteriophora*	*Al-Mearag village (Behera)
MH	*P. luminescens*	*H. bacteriophora*	*El- Dakhla Oasis (El-Wadi El-Gadid)

** Mona and Abou El- Soud (2006), * The present study

proteins in Egyptian *Bt* isolates (Nariman *et al.* 2008).

The present study aims to genetically characterize local *P. luminescens* and *X. nematophila* isolates in order to delineate and identify the genetic relationship between the bacterial symbionts using RAPD-PCr and SDS-PAGE proteins and characterize their toxicity against *Galleria mellonella* larvae using different feeding methods.

MATERIALS AND METHODS

Organisms and growth conditions

Four strains of nematodes were isolated from the soil samples collected in May 2005 from four Egyptian governorates (**Table 1**) using *Galleria* larvae as bait (Woodring and Kaya 1988).

Ten 3rd instar wax moth larvae (*G. mellonella*) were placed into a Petri dish padded with moist Whatman filter paper #1 with 100 dauer juveniles of both nematode strains (*Heterorhabditis bacteriophora* and *Steinernema carpocapsae*) placed per insect. After about 48 h the cadavers were washed in staining block with 70% ethanol for 10 min. Then the cadavers were dissected with a needle and a drop of the hemolymph was streaked on nutrient bromothymol blue agar plates (NBTA), nutrient agar supplemented with 0.025% bromothymol blue and 0.04% triphenyltetrazolium chloride, with a sterile loop (Johnigk *et al.* 2004). The plates were incubated at 28°C in the dark for 48 h then a single colony was selected and transferred to yeast salts (YS) broth (0.5 g $NH_4H_2PO_4$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 5 g NaCl, 5 g yeast extract) and incubated for 2 days at 28°C in the dark (Strauch and Ehlers 1998). Aliquots of 2 ml glycerol at 15% (v/v) were added and stored at -80°C.

Stock inocula of the three *P. luminescens* isolates and *Xenorhabdous nematophila* (BA2 isolate) were produced by inoculating 175 ml of 2% proteose peptone no. 3 (PP3) liquid media (Difco) with a primary variant subclone in a 500 ml flask and incubated for 16 h at 28°C on a rotary shaker at 150 rpm. Production broth was made by inoculating 1.75 ml of the stock inocula into fresh PP3 medium in 500 ml flasks (175 ml of culture/flask). After inoculation, the culture was incubated at 28°C for 24 h as above. Following incubation, the broth was centrifuged at 2,600 × *g* for 1 h at 10°C and vacuum filtered through Whatman GF/D (2.7 Micron) and GF/B (1 Micron) glass filters.

Isolation and propagation of nematodes

EPN were recovered from the soil samples using the insect baiting technique described by Bedding and Akhurst (1975). Insect baits of third-instar *Galleria mellonella* (L.) larvae were placed in 250 ml plastic containers (five samples/containers). Containers were covered with a lid, turned upside down and kept at 27°C. *G. mellonella* larvae were checked every three days and dead larvae were replaced by fresh ones. After seven days, dead insects were rinsed thoroughly in distilled water and placed in modified White traps (Kaya and Stock 1997) until emergence of 3rd stage infective juveniles. Emerging nematodes were pooled for each sample and used to infect fresh *G. mellonella* larvae to produce nematodes for identification and establishment of cultures.

Production of bacterial cell suspensions and their toxicity against 3rd instar larvae of *G. mellonella*

A random bioassay test was done to select the most promising strain to be used in biological control of the insect pest and based upon this test both BA1 and BA2 were chosen to study their pathological effect against wax moth larvae. A single colony of each bacterium was selected and inoculated into 500 ml of nutrient broth no. 2 (Oxoid) and placed in a shaking incubator at 180 rpm for 24 h at 28°C. Based on previous results obtained in a pilot experiment, the concentration of cells used in the experiments was adjusted to 8.35, 4.17, 2.08, 1.04 and (0.52 × 10^3) cells/ml^{-1} for *X. nematophila* and adjusted to 5.35, 2.67, 1.34, 0.67 and (0.33 × 10^3) cells/ml^{-1} for *P. luminescens.* Ringer's solution (a solution of boiled water containing 8.6 g $NaCl_2$, 0.3 g KCl, and 0, 33 g $CaCl_2$ per litre) was added to dilute the bacterial suspension.

Three different methods were used against the 3rd larval instar of *G. mellonella* whereas the bacterial supernatant was injected directly to the hemolymph, mixed with the insect diet and sprayed directly on the larvae. After application with the selected method, cups were sealed and kept in an incubator at 28°C. Experiments were checked over 9 day intervals and the experiment was repeated in triplicate.

Table 2 Primer sequences used for RAPD amplification.

Primers	Names	Primer sequences (5′-3′)
RAPD	UBC-89	GGG GGC TTG G
	UBC-16	GGT GGC GGG A
	UBC-37	CCG GGG TTT T
	UBC-28	CCG GCC TTA A

DNA extraction and RAPD-PCR amplification conditions

The four bacterial isolates were cultured in LB medium overnight at 30°C. Cells were centrifuged at 12,000 rpm for 5 min and the pellets were collected. DNA extraction was performed using the Wizard® SV kit (Promega, Madison, USA). The sizes of the fragments were estimated based on a DNA ladder of 100 to 1500 bp (Bioron GmbH, Germany). Random amplified polymorphic DNA (RAPD) analysis was performed using 10-mers of four UBC random primers obtained from (NAPS Unit, The University of British Columbia, Canada) as shown in **Table 2**.

PCR amplification was performed in a Perkin Elmer 9600 thermal cycler (Martinsburg, West Virginia, USA) in a total volume of 25 µl containing 50 ng DNA, 0.25 mM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ and 1.25 U *Taq* DNA polymerase (Promega). RAPD-PCR was performed under the following conditions: 2 min at 95°C and then 30 cycles of 1 min 94°C, 1 min at 40°C and 2 min at 72°C. The final extension was carried out for 5 min at 72°C. Amplification products were separated using 1% agarose gel electrophoresis in 1% TBE buffer and stained with 0.2 µg/ml ethidium bromide and were visualized using Gel Doc XR system (Bio-Rad laboratories Inc., CA, USA).

SDS-PAGE of cell proteins

Proteins of the four bacterial isolates were obtained according to Von Tersch and Gonzalez (1994) as follows. Cells were cultured in LB medium overnight at 30°C then were centrifuged at 12,000 rpm for 15 min. The pellets were collected and washed twice with high salt TNT buffer (50 mM Tris-HCl pH 7.5, 1.0 M NaCl, 0.05% Triton X-100) followed by two washes with low salt TNT buffer (100 mM NaCl). Aliquots were heated in Laemmli buffer (10% (w/w) glycerol, 5% (w/w) β-mercaptoethanol, 1% (w/v) SDS, 0.188 M Tris-HCl pH 6.8, 0.01% (v/v) bromphenol blue) at 100°C for 5 min. The solubilized proteins were size fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE). After size fractionation, the proteins were visualized by staining with Coomassie Blue R-250 dye. The gel was destained with a large excess of destaining solution (40: 10: 50 of methanol, acetic acid and water). The destaining solution was changed several times until the background become clear.

Genetic analysis

RAPD and protein bands were scored as present (+) or (-) absent. The data were used for similarity-based analysis using the program MVSP (Ver. 3.1b) from www.kovcomp.com. RAPD and protein analyses were analyzed using Nei's genetic similarity index (Nei and Li 1979), where similarity = $2N_{ab}/(N_a + N_b)$ where N_{ab} = number of scored amplified fragments with the same molecular size shared between a and b, and N_a and N_b = number of scored amplified fragments in a and b, respectively. A dendrogram was constructed based on the similarity matrix data by unweighted pair-group method, arithmetic average (UPGMA) cluster analysis.

RESULTS

Toxicity of the local isolates against 3rd instar larvae of *G. mellonella*

All concentrations of direct spray with *X. nematophila* gave reasonable value percentages except for the lowest concentration (0.52×10^3) after 48 and 27 h that gave zero values similar to the control. 60% mortality in the 3rd instar larvae of *G. mellonella* was recorded 4 days after application with 8.35×10^3 cells/ml. When the same bacteria were mixed with the diet of the wax moth larvae (**Table 3**), 60% mortality was observed after 5 days feeding on treated food with the following concentrations: 8.35×10^3, 2.08×10^3 and 1.04×10^3 cells/ml. Meanwhile, only 20% mortality was obtained at a concentration of 0.52×10^3 cells/ml.

Feeding the 3rd instar larvae on media treated with five concentrations of *P. luminescens* recorded 66.6, 60, 46.6 and 13.3% after 9 days of application with 5.35×10^3, 2.67×10^3, 1.34×10^3 and 0.67×10^3 cells/ml, respectively (**Table 4**). However, direct spray of the bacterial cells on *Galleria* larvae caused 73.33% mortality at 5.35×10^3 cells/ml after 8 days of treatment (**Table 4**).

When *X. nematophila* cells were injected directly into the hemolymph the 3rd instar larvae of *G. mellonella*, all insects died after 48 h of injection. However, only *P. luminescens* BA1 displayed a similar result after 48 h and the other two isolates (BAA1 and MH) after 72 h (**Table 5**).

Genetic characterization of the local isolates by RAPD analysis

A total of 68 DNA fragments ranging in size from 7.25 to 0.15 kb were amplified, 46 of which were polymorphic while the other amplified fragments were common among the four isolates. The mean percentage polymorphism shown by the four primers was 67.6%, specifically decreasing in this order: UBC-37 (75%), UBC-89 (73.3%), UBC-16 (65%) and UBC-28 (58.8%) (**Table 6**). Among the 46

Table 3 Mortality percentages after subsequent feeding hours of *Xenorhabdus nematophila* conc. on third *Galleria mellonella* larval instars using direct spray and feeding methods.

Strain	Conc. (cell/ml)	Direct feeding				Direct spray			
		48	72	96	120 h	48	72	96	120 h
BA2	8.35×10^3	0.00	33.3	40.0	60.0	30.0	40.0	60.0	60.0
	4.17×10^3	0.00	33.3	40.0	53.3	20.0	40.0	40.0	50.0
	2.08×10^3	0.00	40.0	46.6	60.0	30.0	50.0	50.0	60.0
	1.04×10^3	0.00	0.00	26.6	60.0	20.0	30.0	40.0	60.0
	0.52×10^3	0.00	0.00	6.60	20.0	0.00	0.00	10.0	20.0

Table 4 Mortality percentages after subsequent feeding days of *Photorhabdus luminescens* conc. on third *Galleria mellonella* larval instars using direct spray and direct feeding methods.

Strain	Conc. (cell/ml)	Direct feeding (days)			Direct spray (days)			
		3	6	9	2	4	6	8
BA1	5.35×10^3	26.6	46.6	66.6	26.7	33.3	60.0	73.3
	2.67×10^3	26.6	33.3	60.0	13.3	20.0	40.0	46.7
	1.34×10^3	13.3	40.0	46.6	0.00	20.0	26.7	26.7
	0.67×10^3	0.00	13.3	13.3	0.00	6.70	13.3	13.3
	0.33×10^3	0.00	0.00	13.3	0.00	0.00	6.70	6.70
BAA1	5.35×10^3	20.0	40.0	46.6	20.0	30.0	60.0	60.0
	2.67×10^3	26.6	33.3	33.3	13.3	33.3	40.0	60.0
	1.34×10^3	13.3	40.0	40.0	0.00	33.3	40.0	53.3
	0.67×10^3	0.00	13.3	13.3	0.00	40.0	46.6	46.6
	0.33×10^3	0.00	0.00	3.30	0.00	0.00	20.0	30.0
MH	5.35×10^3	6.70	26.6	60.0	13.3	33.3	60.0	66.6
	2.67×10^3	3.30	33.3	40.0	3.30	13.3	46.7	46.7
	1.34×10^3	0.00	33.3	33.3	0.00	20.0	26.7	26.7
	0.67×10^3	0.00	13.3	13.3	0.00	3.30	13.3	13.3
	0.33×10^3	0.00	0.00	13.3	0.00	3.30	3.30	6.70

Table 5 Mortality percentages after different feeding hours of *X. nematophila* and *P. luminescens* concentrations on third *Galleria mellonella* larval instars by direct injection into the hemolymph.

Conc. (cell/ml)	*X. nematophila*			*P. luminescens*								
	BA2			BA1			BAA1			MH		
	24	48	72 h	24	48	72 h	24	48	72 h	24	48	72 h
8.35×10^3	100	100	-									
4.17×10^3	100	100	-									
2.08×10^3	100	100	-									
1.04×10^3	80	100	-									
0.52×10^3	70	100	-									
5.35×10^3				100	100	-	80	10	100	10	80	100
2.67×10^3				100	100	-	65	10	100	10	75	100
1.34×10^3				100	100	-	50	40	100	10	60	100
0.67×10^3				90	100	-	40	30	100	10	40	100
0.33×10^3				70	100	-	30	50	100	5	40	100

The untreated four isolates (Controls) of the direct feeding, spray and injection were recorded zero values after all different feeding days and hours.

Table 6 RAPD analysis of the four local isolates using four random primers.

Primer name	Polymorphic Percentage of total bands	Band No.	Band size (kb)	*P. luminescens*			*X. nematophila*	Polymorphic percentage of specific bands
				BA1	BAA1	MH	BA2	
Primer UBC-89	73.3	1	6.74	+	+		+	13.3
		2	3.14			+	+	
		3	2.41			+	+	
		4	1.87				(+) Sp	
		5	1.63			+	+	
		6	1.41		+	+	+	
		7	1.30				(+) Sp	
		9	0.92		+	+		
		10	0.78		+	+	+	
		12	0.56	+		+	+	
		13	0.49	+	+			
		Total= 15	11	3	5	7	9	
Primer UBC-16	65	1	7.25	+	+		+	20
		2	3.45	+	+			
		5	2.44		+	+	+	
		7	1.83	(+) Sp				
		8	1.64	+	+	+		
		10	1.28	+	+			
		11	1.21		+	+	+	
		15	0.67	+	+		+	
		16	0.60	+	+	+		
		17	0.45			(+) Sp		
		18	0.40	+	+			
		19	0.31			(+) Sp		
		20	0.28		(+) Sp			
		Total= 20	13	8	10	6	4	
Primer UBC-37	75	1	5.00		+	+		12.5
		2	3.48	+	+	+		
		3	2.90	+	+	+		
		5	1.66		+	+		
		7	1.31	+	+			
		8	1.09	+	+	+		
		9	0.97				(+) Sp	
		11	0.77	+	+	+		
		12	0.65		+		+	
		14	0.43				(+) Sp	
		15	0.40		+	+		
		16	0.34	+	+	+		
		Total= 16	12	6	10	8	3	
Primer UBC-28	58.8	1	3.42		+		+	12
		2	2.66		+	+		
		4	1.89		+		+	
		8	0.77	+		+	+	
		10	0.57				(+) Sp	
		12	0.40		+	+	+	
		13	0.34				(+) Sp	
		15	0.26	+		+		
		16	0.22	+	+	+		
		17	0.15	+		+		
		Total= 17	10	4	5	6	6	
Polymorphism=67.6%		68	46	21	30	27	22	14.7%

+ = Presence of fragment, Total = Total number of amplified fragments, Sp = Isolate-specific fragment

polymorphic fragments, 8 were isolate-specific observed only in three isolates, BA2, BA1 and BAA1. BA2 had the highest number (6) of specific fragments and both BA1 and BAA1 had one each.

The total number of amplified fragments of the four isolates varied considerably. For instance, BA2, belonging to *X. nematophila*, revealed the highest (9) with primer UBC-89 and the lowest (4 and 3) with primers UBC-16 and UBC-37. Among the three *P. luminescens* isolates, BAA1 showed the highest (10) using primers UBC-16 and UBC-37 and BA1 revealed the lowest (3 and 4) using primers UBC-89 and UBC-28. Primer UBC-89 revealed 15 fragments, 11 of which were polymorphic with sizes ranging from 6.74 to 0.49 kb (**Fig. 1, Table 6**). Among the four isolates, two unique fragments (1.87 and 1.3 kb) were observed in BA2, and three similar fragments (3.14, 2.41 and 1.63 kb) characterized BA2 and MH while two (1.41 and 0.78 kb) were present in the three isolates but not in BA1. The residual fragments were distributed in either two different isolates or three. Primer UBC-16 revealed 20 fragments, 13 of which were polymorphic with sizes ranging from 7.25 to 0.28 kb (**Fig. 1, Table 6**). Four fragments were uniquely displayed in three isolates: MH displayed two (0.45 and 0.31 kb) and BA1 and BAA1 displayed one each (1.83 and 0.28 kb, respectively). Similar fragments were existed and characterized some isolates, such as three (3.45, 1.28 and 0.4 kb) in BA1 and BAA1, two (7.25 and 0.67 kb) in BA1, BA2 and BAA1 and two (2.44 and 1.21 kb) in BA2, BAA1 and MH. Primer UBC-37 revealed 16 fragments, 12 of which were polymorphic with sizes ranging from 5.0 to 0.34 kb (**Fig. 1, Table 6**). BA2 was characterized by two unique fragments (0.43 and 0.97 kb) that were absent in the other three isolates. However, this isolate did not have five amplified fragments (3.48, 2.9, 1.09, 0.77 and 0.34 kb) that were present in the other three isolates. Three similar fragments (5, 1.66 and 0.4 kb) were unique to both BAA1 and

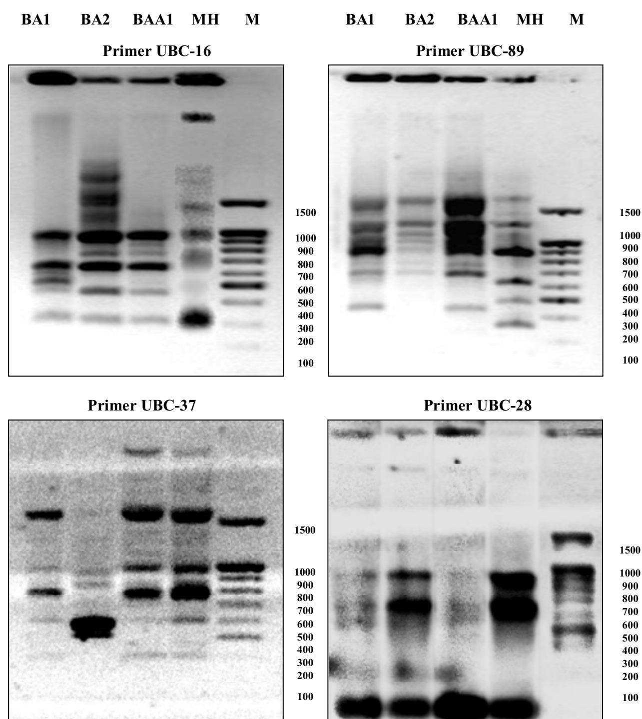

Fig. 1 RAPD amplification profiles of the four local isolates using four random primers. M= 1 kb DNA ladder with 11 bands.

MH. Primer UBC-28 revealed 17 amplified fragments, 10 of which were polymorphic with sizes ranging from 3.42 to 0.15 kb (**Fig. 1, Table 6**). BA2 of *X. nematophila* was characterized by two unique fragments (0.34 and 0.57 kb) that were not present in the other three isolates. Two fragments (0.26 and 0.15 kb) were unique to both BA1 and MH while two others (3.42 and 1.89 kb) were unique in the other two isolates (BA2 and BAA1).

In general, among the 46 polymorphic fragments, 8 isolate-specific fragments were detected in the four isolates. BA2 belonging to *X. nematophila* revealed 6 specific bands with the 3 UBC primers: UBC-89 (1.87, 1.30 kb), UBC-37 (0.97, 0.43 kb) and UBC-28 (0.57, 0.34 kb) while MH, belonging to *P. luminescens*, displayed two (0.40 and 0.31 kb) with UBC-16 and BA1 and BAA1 each showed one specific band with one primer: UBC-89 (1.83 kb) in BA1 and UBC-16 (0.28 kb) in BAA1.

Genetic characterization of the four isolates by SDS-PAGE protein analysis

SDS-PAGE analysis of total proteins extracted from the four isolates revealed 45 bands with different molecular weights ranging from 283 to 10 kD, as shown in **Fig. 2** and **Table 7**. Among these, 34 protein bands varied distinctly in some isolates, while the other 11 bands were commonly among all four isolates. The remarkably large genetic variation between the four isolates occurred as a consequence of different protein bands that were not located in their fitting position on the gel like the other bands in the isolates, but they shifted up or down with different molecular weights. For instance, a 119 kD band was unique to BA2, while band no. 10 with 116 kD existed in the other three isolates but not in BA2. The 106 kD band in BA2 showed a similar trend relative to the common band no 11 with 112 kD. Band no. 39 (40 kD) was shifted on the gel in BA2 from the common band no. 40 with 33 kD. Three bands (54, 41 and 39 kD) revealed the similar notice in BAA1. Moreover, band no. 27 (61 kD) was altered its position on the gel from the common band no. 26 with 63 kD in MH.

Genetic characterization of the four isolated strains comprised of three *P. luminescens* (BA1, BAA1, MH) and *X. nematophila* (BA2) revealed several specific protein bands that appeared in each isolate uniquely and disappeared in the other isolates. For example, one band with 50 kD was detected separately in BA1 and disappeared in the other three isolates, seven bands with different molecular weights were specific to BA2, three bands with 54, 41 and 39 kD in BAA1 and two bands with 67 and 61 kD were existed uniquely in MH. Moreover, two different isolates showed similar bands that were not existed in the other two isolates, such as 56 kD in (BA1 and BA2), 142 kD in (BA1 and BAA1), three bands with 168, 37 and 10 kD in (BA1 and MH). In addition, 75 kD in (BAA1 and BA2) and 44 kD in (BAA1 and MH). It is interesting to note that, each isolate was characterized by disappearence of one band that was existed in other isolates, for instance two (65 and 148 kD) in BA1, seven in BA2, two (43 and 28 kD) in BAA1 and three (63, 58 and 27 kD) in MH.

Table 7 SDS-PAGE analysis of the polymorphic protein bands of the four isolates.

Band presence in isolates	Band No.	Mw (kD)	*P. luminescens*			*X. nematophila*
			BA1	BAA1	MH	BA2
One band in one isolate	6	131				+
	9	119				+
	12	106				+
	16	89				+
	18	82				+
	21	74				+
	39	40				+
	24	67			+	
	27	61			+	
	30	54		+		
	36	41		+		
	38	39		+		
	32	50	+			
In two isolates	2	168	+		+	
	43	37	+		+	
	45	10	+		+	
	4	142	+	+		
	20	75		+		+
	29	56	+			+
	34	44		+	+	
In three isolates	1	283	+	+	+	
	10	116	+	+	+	
	11	112	+	+	+	
	15	93	+	+	+	
	19	78	+	+	+	
	23	68	+	+	+	
	40	33	+	+	+	
	25	65		+	+	+
	3	148		+	+	+
	26	63	+	+		+
	28	58	+	+		+
	44	27	+	+		+
	35	43	+		+	+
	37	28	+		+	+
In four isolates	5	138	+	+	+	+
	7	126	+	+	+	+
	8	123	+	+	+	+
	13	101	+	+	+	+
	14	96	+	+	+	+
	17	86	+	+	+	+
	22	70	+	+	+	+
	31	52	+	+	+	+
	33	47	+	+	+	+
	41	32	+	+	+	+
	42	30	+	+	+	+

+ = Presence of protein bands

Genetic similarity of the four isolates using RAPD and SDS-PAGE protein analyses

Genetic similarity between each two pairs of the four isolates was performed using Nei's similarity index based on RAPD amplified fragments and the polymorphic protein

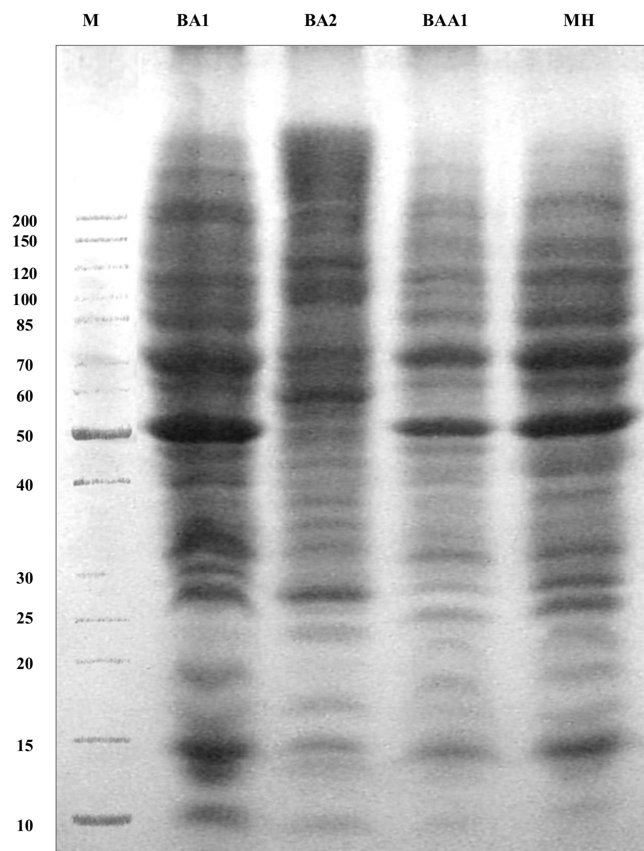

Fig. 2 SDS-PAGE protein profiles of the four isolates. M= Protein marker with molecular weights (kD).

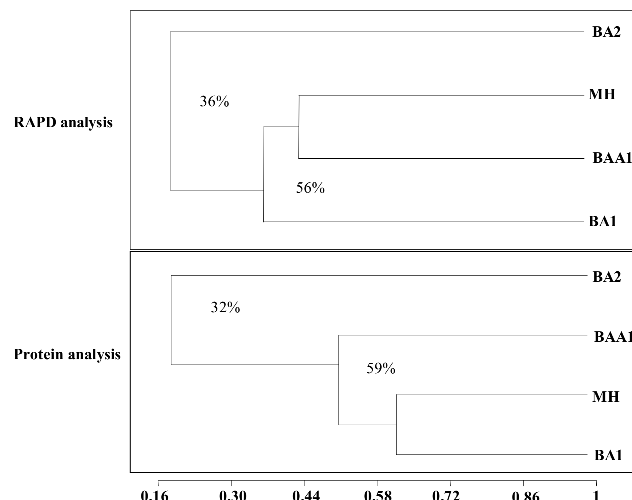

Fig. 3 dendrogram represented the genetic relationships among the four isolates using UPGMA cluster analysis of Nei's genetic similarity coefficients generated from four RAPD primers and protein analysis.

bands as presented in **Table 8**. Genetic similarity between the four isolates was calculated from the amplified fragment data using unweighted pair group method with arithmetic average (UPGMA). Two dendrograms were constructed using the four RAPD primers and protein bands (**Fig. 3**). The dendrogram of the four RAPD primers obviously indicates two main clusters. BA2 belonging to *X. nematophila* is the first major cluster and the second includes the three other isolates (BA1, BAA1 and MH) belonging to *P. luminescens* and it has two subclusters. Whereas, the first include BAA1 and MH that showed the highest genetic relationship with 63.2%, comparing with the other three isolates. The second subcluster is contained the first subcluster and BA1 with bootstrap of 56%. the UPGMA dendrogram of the polymorphic protein bands revealed similar two clusters like the RAPD. Whereas, the first was BA2 and the second cluster contained the three isolates that was divided into two distinct subclusters; MH and BA1 with genetic similarity value of 68.6%. BAA1 is the major second subcluster with bootstrap 59% with the first subcluster (MH and BA1).

In general, it is clearly noticed that genetic similarity percentages are extremely low, not as expected, between BA2 (*X. nematophila*) and the three isolates BA1, BAA1 and MH (*P. luminescens*) as well as between the three isolates either in RAPD or protein analysis (**Table 8**). Hence, combining the UPGMA data of RAPD-PCR with protein analysis could provide a valuable and reliable tool for discriminating between different bacterial isolates. Furthermore, development of this simple molecular approach could be very informative and beneficial, particularly with respect to screening procedures.

DISCUSSION

Steinernema and *Heterorhabditis* nematodes have been used to control a wide range of agriculturally important insect pests (Hajek *et al.* 2007). The mechanism by which these nematodes are able to infect and reproduce in the insect host involves a mutual relationship between the nematode and the symbiotic bacteria, *Xenorhabdus* and *Photorhabdus*. Nematodes infect insect larvae and release the colonized bacteria into the insect blood (Nawaz *et al.* 2005). The bacteria kill the insect larvae and convert the cadaver into a food source suitable for nematode growth and development. However, in the present study the four bacterial isolates (BA1, BA2, BAA1 and MH) were directly fed and sprayed as well as injected into the hemolymph of *G. mellonella* larvae without their host nematodes.

In **Tables 3-5**, the four isolates revealed high mortality that reached 100% after the bacteria was injected into the hemolymph, while strain BA2 of *X. nematophila* showed 60% mortality using direct feed and spray. The three other isolates of *P. luminescens* displayed mortality with 66.6 and 73.3% using direct feed and spray, respectively. The results were confirmed by Bowen and Ensign (1998), who grew *P. luminescens* in peptone broth; in the absence of the nematodes, the bacteria produced a protein toxin complex that was lethal when fed to, or injected into the hemolymph of, *Manduca sexta* larvae and several other insect species. They reported that the toxin purified as a protein complex that had an estimated molecular weight of 1,000,000 and contained no protease, phospholipase or hemolytic activity and only a trace of lipase activity. Jung and Kim (2006) reported that *Xenorhabdus* and *Photorhabdus* could be applied to kill

Table 8 Genetic similarity percentages of the four isolates based on RAPD products of four primers and protein analysis.

Methods	Local isolates	BA1	BA2	BAA1	MH
RAPD-PCR	BA1	100			
	BA2	23.3	100		
	BAA1	62.7	42.3	100	
	MH	50.0	40.8	63.2	100
SDS-PAGE proteins	BA1	100			
	BA2	35.3	100		
	BAA1	61.1	35.3	100	
	MH	68.6	24.2	57.1	100

Spodoptera exigua by oral treatment in a mixture with *Bt aizawai.* Considering the fact that both nematode-symbiotic bacteria usually infect the hemocoel of target insects only after the delivery by the infective juveniles (Akhurst 1980; Boemare *et al.* 1997), the oral pathogenicity may be explained by direct toxic action in the gut lumen.

Photorhabdus and *Xenorhabdus* bacteria engage in both pathogenic and mutualistic interactions with different invertebrate hosts as obligate components of their life cycle (Goodrich-Blair and Clarke 2007). Therefore, the bacteria have a complex life cycle that involves temporally separated pathogenic and mutualistic associations with two different invertebrate hosts. This tripartite *Photorhabdus*-insect-nematode association provides researchers with a unique opportunity to characterize the prokaryotic contribution to two different symbioses, i.e. pathogenicity and mutualism (Clarke 2008).

Moreover, one of the pathogenic mechanisms of *Photorhabdus* and *Xenorhabdus* bacteria includes host immunodepression, which leads to lethal septicemia. It has been known that *X. nematophila* inhibits phospholipase A2 (PLA2) to induce host immunodepression. Kim *et al.* (2005) tested the hypothesis of PLA2 inhibition using another bacterial species involved in other genera. *P. temperata* subsp. *temperata* is the intestinal symbiont of an entomopathogenic nematode, *H. megidis*. The bacteria caused potent pathogenicity in a dose-dependent manner against the 5th instar larvae of *Spodoptera exigua*, as early as 24 h after the intra-hemocoelic injection. In response to the live bacterial injection, hemocyte nodulation (a cellular immune response) and prophenoloxidase (pPO) activation were inhibited, while the injection of heat-killed bacteria significantly induced both immune reactions. The immunodepression induced by the live bacteria was reversed by the addition of arachidonic acid, the catalytic product of phospholipase A2. In contrast, the addition of dexamethasone, a specific PLA2 inhibitor to the heat-killed bacterial treatment, inhibited both immune capacities.

In addition to a previously known PLA2 inhibitory action of *X. nematophila*, the inhibition of *P. temperata* on PLA2 suggests that bacteria symbiotic to entomopathogenic nematodes share a common pathogenic target to result in an immunodepressive state of the infected insects. To prove this generalized hypothesis, we used other bacterial species (*X. bovienni*, *X. poinarii*, and *P. luminescens*) involved in these two genera. All our experiments clearly showed that these other bacteria also share their inhibitory action against PLA2 to induce host immunodepression.

Out of 46 *Photorhabdus* isolates and six *Xenorhabdus* isolates only six North American *P. temperata* isolates were toxic to the thrips species. After 7 days of drinking from *P. temperata* supernatant a mortality of 90% could be reached. Thrips were also killed after sucking from leaves covered with the toxins (Gerritsen *et al.* 2005). Moreover, *Photorhabdus temperata* strain K122 exhibited oral toxicity against *Prays oleae* (Tounsi *et al.* 2006).

Classic microbiological methods are generally not suitable, because they do not distinguish the biocontrol agent strains in the natural microbiota. However, genotypic markers are preferable because they are more stable and their expression does not depend on the type of culture media used for analysis.

Getting specific genotypic markers is a difficult, time-consuming process that can be accomplished by DNA fingerprinting of the biocontrol agent strains. These methods are based on the amplification of gene sequences by means of RAPD-PCR and by comparing the fingerprint patterns of the biocontrol isolate strains; strain-specific fragments can be identified (Montesinos 2003). In our study, RAPD-PCR analysis (**Table 6**) demonstrate genetic divergence between the three local isolates of *P. luminescens* (BA1, BAA1 and MH) and *X. nematophila* (BA2), whereas fragments with distinctive sizes (kb) were not detected in BA2 and clearly observed in the three others. RAPD analysis was genetically discriminated between the three isolates (BA1, BAA1 and MH) uniquely by the absent of fragments in one and present in the two other isolates. More characterization was obtained between *X. nematophila* (BA2) and the three *P. luminescens* isolates (MH, BA1 and BAA1) using the isolate-specific fragments, which emphasized that each isolate has a unique pattern and clearly differentiate *X. nematophila* and *P. luminescens.* More evidence was obtained from UPGAMA clustering of RAPD and protein analyses, which confirmed that BA2 has a separate cluster outlying from the second cluster containing the three other isolates.

Our results were confirmed from several reports, for instance Fischer-Le Saux *et al*. (1998) identified thirty distinctive 16S rDNA genotypes from a collection consisted of 77 isolates recovered from entomopathogenic nematodes and of 40 reference strains belonging to *Xenorhabdus* and *Photorhabdus* spp. collected at various localities worldwide. They used UPGAMA cluster analysis to distinguish the genus *Xenorhabdus* from the genus *Photorhabdus*. Their results showed that genus *Xenorhabdus* appears more diverse than the genus *Photorhabdus*, and for both genera the bacterial genotype diversity is in congruence with the host-nematode taxonomy.

In both genera, identification of new bacterial isolates is difficult (Boemare and Akhurst 1988) because most strains are phenotypically very similar and fail to give positive results in many classical tests for identification and because of a lack of sufficient members per taxon. Consequently, only a few species have been described, and some of these are represented by only a few isolates (Akhurst and Boemare 1988). Ecological data relating bacterial symbionts with their nematode host or their environment remain weak. Thus, studies on the taxonomic diversity and distribution of members of *Xenorhabdus* and *Photorhabdus* spp. are needed. Using inexpensive techniques such as RAPD marker and SDS-PAGE protein, typing several isolates can be performed rapidly with an opportunity to find new isolate patterns also the distribution of the isolate genotypes could be studied in relation to geographical and nematode sources. Consequently, the present results confirming the taxonomic heterogeneity of the four isolate belonging to *X. nematophila* and *P. luminescens*. On the other hand, SDS-PAGE analysis (**Table 7**) revealed 45 protein bands in the three *P. luminescens* isolates (BA1, BAA1, MH) and in *X. nematophila* (BA2). Among them, several bands with different molecular weights were reported to be toxic and exhibited insecticidal activity against many insects. For instance, the identified protein with 40 kD in BA2 isolate only was purified by Ensign *et al.* (1990) as a toxin molecule from *X. luminescens* NC-19. Khandelwal and Banerjee-Bhatnagar (2003) found that such protein extracted from live cells of *X. nematophia* and the outer membrane vesicle derived from them exhibited oral insecticidal activity against *Helicoverpa armigera* neonatal larvae. Toxin A protein with 283 kD that identified in BA1, BAA1 and MH was reported by Liu *et al.* (2003) as a product of *tcdA* gene of *P. luminescens* and is highly toxic to a variety of insects, such as tobacco hornworm (*Manduca sexta*) and southern corn rootworm (*Diabrotica undecimpunctata howardi*). The protein of 37 kD detected in BA1 and MH was identified by Kiara *et al.* (2007) as a secreted protein in various *Photorhabdus* strains that identified as the product of the *prtS* gene and stimulated a rapid melanization reaction when injected into *M. sexta*, *Galleria mellonella* and *Drosophila melanogaster* larvae.

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