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Novel Characteristics of Local *Xenorhabdus* and *Photorhabdus* Isolates with Phenotypic Heterogeneity, 16S rRNA Sequence Variation and High Toxicity to *Galleria mellonella*

Nariman A. H. Aly[1*] • Hussein A. Mona[2]

[1] Microbial Genetics Department, National Research Center, Cairo, Egypt
[2] Pests and Plant Protection Department, National Research Center, Cairo, Egypt

Corresponding author: * **nariman_mongy@yahoo.com**

ABSTRACT

Four local symbiotic bacterial isolates comprised of three *Photorhabdus luminescens* and one *Xenorhabdus nematophila* isolated from nematodes (*Heterorhabditis bacteriophora* and *Steinernema carpocapsae*, respectively) that were previously characterized by their high toxicity to *Galleria mellonella*, were characterized. The nematodes were isolated from the soil samples collected from four Egyptian governorates using *Galleria* larvae as bait. Cultural properties as well as cellular morphology of primary and secondary form variants are discussed. Four forward primers within the variable domain of the 16S rRNA gene at position 440 to 480 bp and the reverse primer from the highly conserved region at 755 to 795 bp were used to amplify a 355-bp fragment. Primer TRO displayed the fragment in the three *P. luminescens* isolates, while it was absent in the *X. nematophila* isolate. On the contrary, primer TEM showed the fragment in the *X. nematophila* isolate and the gene was undetected in the three *P. luminescens* isolates. The two other primers revealed the fragment in all isolates. Sequence alignment revealed 32 nucleotide positional differences between the novel local *Photorhabdus* isolate (FJ755891) and the numerous isolates and strains of *P. luminescens* and *X. nematophila* based on the 16S rRNA gene similarity. Phylogenetic analysis based on 16S rRNA gene sequences showed that the local isolate (FJ755891) formed a phylogenetically distinct group, separate from all other named isolates and species of *P. luminescens* and *X. nematophila*. Such obtained results evidently indicated a large diversity with unique characteristics of the local Egyptian isolates from all the other isolates and strains established around the world. Two of the four specific primers detected the 16S rRNA gene with 355 bp in the four isolates, while the two other primers displayed the gene in either one and three isolates belonging to *P. luminescens*.

Keywords: Accession FJ755891, local *Xenorhabdus* and *Photorhabdus* isolates, 16S rRNA gene

INTRODUCTION

Xenorhabdus and *Photorhabdus* spp. are two genera of bacteria that symbiotically associate with Steinernematidae and Heterorhabditidae, respectively (Poinar 1990). They are motile, Gram-negative bacteria belonging to the family *Enterobacteriaceae* ((Boemare *et al.* 1993); the general features of the life cycles of these bacteria are quite similar. The similarities include habitation in the gut of entomopathogenic nematodes, growth in the hemolymph of larval stage insects, and pathogenic potential toward the infected insect. The nematode-bacterium pair is capable of invading and killing the larval stage of numerous insects (Akhurst and Boemare 1990). Both *Xenorhabdus* and *Photorhabdus* spp. are carried as symbionts in the intestine of the infective juvenile stage of nematodes. The nematodes enter the digestive tract of the larval stage of diverse insects and subsequently penetrate into the hemocele of the host insect. The nematode can also gain access to the hemocele via the respiratory spiracles or by penetrating directly through the insect cuticle (Akhurst and Dunphy1993). Upon entrance into the hemocoel, the nematodes release the bacteria into the hemolymph. Together, the nematodes and the bacteria rapidly kill the insect larva, although in most cases the bacteria alone are highly virulent.

In both genera, identification of new bacterial isolates or species is difficult because most strains are phenotypically very similar and fail to give positive results in many classical tests for identification (Boemare and Akhurst 1988). Therefore, molecular identification has been popularized to identify or diagnose species for nematodes and bacteria. Thus far, the potency of DNA sequences such as the16S ribosomal RNA gene (rRNA) has been reported for the molecular identification of nematode bacterium entomopathogens (Adams *et al.* 2006). The 16S rRNA gene was used for molecular typing strains and isolates belonging to *Xenorhabdus* and *Photorhabdus* spp. For instance, Fischer Le Saux *et al.* (1999) conducted a polyphasic, comprehensive approach for the description of species within *Photorhabdus*, which included phenotypic characterization, 16S rRNA analysis and examination of DNA relatedness. It was determined that *Photorhabdus* consists of three species: two symbiotic species, *P. luminescens* and *P. temperata*, and one clinical species, *P. asymbiotica*. Comparisons of the 16S rRNA gene sequences of 76 *Xenorhabdus* species isolated from 27 species of *Steinerema* nematodes and collected in 32 countries identified 13 groups and seven unique sequences. The classification of the strains lead to classify new isolated into *Xenorhabdus* species and description of ten novel species (Tailliez *et al.* 2006). Fischer Le Saux *et al*. (1998) studied the genetic diversity of 77 isolates recovered from entomopathogenic nematodes in 14 Caribbean islands and of 40 reference strains belonging to *Xenorhabdus* and *Photorhabdus* spp. collected at various localities worldwide. Thirty distinctive 16S rRNA genotypes were identified and the genus *Xenorhabdus* appears more diverse than the genus *Photorhabdus*. For both genera, the bacterial genotype diversity is in congruence with the host nematode taxonomy and the occurrence of symbiotic bacterial genotypes was related to the ecological distribution of host

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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 (Al-Behera)
MH	*P. luminescens*	*H. bacteriophora*	*El- Dakhla Oasis (Al-Wadi Al-Gadid)

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

nematodes. The phylogenetic analysis of the symbiotic bacteria isolated from Japanese heterorhabditid nematodes based upon the 16S rRNA gene sequences indicated that all the bacterial isolates associated with Japanese *H. megidis* were *P. temperate*. On the other hand, two types of symbiotic bacteria were isolated from Japanese *H. indica*. *Photorhabdus luminescens* subsp. *akhurstii* is known as the symbiotic bacterium that associates with *H. indica* (Boemare 2002). However, in another study by Kuwata *et al.* (2007), two isolates from two Japanese *H. indica* isolates formed a cluster with *P. asymbiotica* subsp. *australis*. A detailed genetic analysis at the species level provides insight into the variability within a bacterial population because certain isolates tend to exhibit unusual phenotypic characteristics and this helps to generate evidence of genome plasticity and evolution, which enable bacterial adaptation to various environmental conditions (Bhattacharya *et al.* 2003).

The present study aims to characterize the new local *Photorhabdus luminescens* and *Xenorhabdus nematophila* isolates that expressed high toxicity against *Galleria mellonella* larvae using conventional morphological methods and 16S rRNA nucleotide sequence alignment.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The three new isolates (BA1, BAA1 and MH) of *P. luminescens* isolated from nematode *H. bacteriophora* and one isolate (BA2) of *X. nematophilaa* isolated from the nematode *S. carpocapsae* were used in the study and their geographical and nematode host sources are shown in **Table 1**.

Nematode isolation and propagation

Entomopathogenic nematodes (EPN) were recovered from the soil samples using the insect baiting technique described by Bedding and Akhurst (1975). Insect baits of five last-instar *Galleria mellonella* (L.) larvae were placed in 250 ml plastic containers (five containers/sample) with moistened soil obtained from each sample. Containers were covered with a lid, turned upside down and kept at 27°C. Water was added to samples if they appear dry at any point during their bating. *G. mellonella* larvae were checked every two to 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 third-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.

Isolation and maintenance of the bacteria

Symbiotic bacteria isolate strains were obtained by two methods from the third-stage infective juveniles (dauer) of BA1, BA2, BAA1 and MH. They were freshly harvested from *Galleria* white traps, collected and washed by centrifugation with sterilized tap water three times. The first method was to crush *ca*. 100 surface-disinfected, followed by streaking the product on nutrient bromothymol blue agar plates (NBTA) (Akhurst 1980).

The second method was to streak onto NBTA plates a drop of hemolymph harvested from infected *Galleria* parasitized by different strains of nematodes. In this method, 10 6th instar wax moth larvae *G. mellonella* were put into a Petri dish padded with moist filter paper with approx. 100 dauer juvenile per insect and the humidity was adjusted to 15%. After about 48 h the cadaver washed in staining block with 70% alcohol for 5-10 min. Then the cadavers were dissected with a needle and a drop of the hemolymph was streaked on NBTA agar with sterile loop. The plates were incubated at 28°C in the dark for 48 h then a single colony was selected and streaked on NBTA, MacConkey and nutrient agar. Colony were then transferred to YS broth and incubated for 1-3 days 28°C in the dark. A 15% of sterile glycerol was added to the bacterial suspension and caps filled with the bacteria were stored at -80°C. To isolate both of the form variants (primary and secondary) distinct colonies were selected from subcultures of the primary form and cultured separately.

Phenotypic characterization of the bacterial symbionts

Conventional morphological criteria were used to verify generic identity (*Xenorhabdus* and *Photorhabdus*) of bacterial isolates (Boemare and Akhurst 1988). Cultural properties such as colony size, shape and color were determined after 3 days incubation at 28°C on nutrient agar medium. All tests were conducted at 28°C in a dark room. Cellular morphology was assessed by microscopic examination of 24 h old nutrient broth cultures using an Olymbous microscope with 10, 20, 40 and 100 X differential interference contrast lens. Dye adsorption of bromothymol blue was tested on nutrient agar supplemented with 0.004% triphenyltetrazolium chloride and 0.0025 bromothymol blue (NBTA medium) for *Xenorhabdus* isolates. Dye adsorption of neutral red was tested on MacConkey agar for *Photorhabdus* isolates. Bioluminescence (the emission of light) was investigated by observing cultures on nutrient agar plates with the naked eye in a darkroom for up to 20 min. The presence of inclusion bodies were detected after 48 h post inoculation of 2 ml bacteria in YS medium.

DNA extraction and PCR amplification of 16S rRNA gene

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 100 to 1500 bp ladder (Bioron GmbH, Germany). According to Ehlers and Niemann (1998), four forward primers within the variable domain of the 16S rRNA gene at position 440 to 480 bp (TYPE, TEM = TEMPERATUS, TRO = TROPICUS and PÜTZ) and the reverse primer from the highly conserved region at 755 to 795 bp were used to amplify a 355-bp fragment. The sequences of the designed primers for 16S rRNA gene are presented in **Table 2**. Amplification was performed in a thermal cycler 9600 Perkin Elmer (Martinsburg, West Virginia, USA) in a total volume of 25 µl containing 50 ng DNA, 0.25 mM each primer, 0.2 mM (each) dNTPs, 1.5 mM $MgCl_2$ and 1.25 U *Taq* DNA polymerase (Promega). PCR was performed for 16S rRNA gene under the following conditions: 5 min at 95°C and then 35 cycles of 1 min 95°C, 1 min at primer specific annealing temperature and 1 min at 72°C. The final extension was carried out for 5 min at 72°C.

16S rRNA gene purification, sequencing and analysis

PCR product of 335 bp was purified with the QIA quick PCR Purification Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. DNA was eluted in 20 µl of sterile water. The

Table 2 Forward and reverse primer sequences used for 16S rRNA gene amplification.

Primers	Names	Primer sequences (5′-3′)	Annealing	Position	Application
16S rRNA gene	(F)TYPE	CAGCGGGGAGGAAGGGTTCA GCTTGAACAGAGCTGAATTTT	68°C	440-480	PCR
	(F)TEM	CAGCGGGGAGGAAGGGTTTA GCCTGAACAGGGTTGAATTTT	68°C		PCR+ sequencing
	(F)TRO	CAGCGGGGAGGAAGGGTTGA GCCTGAACAGGGCTGGGCCTT	65°C		PCR + sequencing
	(F)PÜTZ	CAGCGGGGAGGAAGGGTCCA GCCTGAAGAGGGTTAGACTTT	59°C		PCR
	(R)	CGAGTCCACGCTTTCGCACC CCTCGTTTGTCCTAATCTATG		755-795	

(F and R) refereed to forward and reverse primers.

16S rRNA fragment was sequenced on an Applied Biosystems automatic sequencer (ABI PRISM® 1200 DNA Sequencer, Bioron GmbH, Germany).

Sequences were compared with sequences of representatives of the most related *Photorhabdus* and *Xenorhabdus* strains deposited in GenBank, EMBL, and sequencing-genome databases by using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). Analysis was performed using Geneious Pro 4.5.4 program. A phylogenetic tree was supported from 500 bootstrap replicates and a dendrogram was constructed using multiple alignment of the 16S rRNA from *P. luminescens* and *Xenorhabdus* isolates and strains.

Nucleotide sequence accession number

The GenBank accession number for the partial nucleotide sequence of the 16S rRNA gene from *P. luminescens* isolate BAA1 is FJ755891.

RESULTS

Phenotypic characterization of the symbionts bacterial isolates

The primary phase cells of the four local isolates were investigated, whereas three isolates (BA1, BAA1 and MH) of *P. luminescens*, the associated bacterial symbionts of the nematode *Heterorhabditis bacteriophora*, almost had the same measures and features. Colonies were circular, convex, their width ranged from 1.5 to 2 µm and they ranged from 3 to 4 µm in length. Further examination of the primary cells showed that they have granulated and opaque cell walls with irregular margins while the secondary form variants are flat, translucent with a regular margin whose width ranged from 1 to 1.5 µm and usually with greater diameter (about 5-7.5 µm) than the primary cells. The primary phase cells of isolate (BA2) belonging to *X. nematophila*, the associated bacterial symbiont of the nematode *Steinernema carpocapsae*, had large colonies (5-8 µm) olive-blue in color and uneven or irregular margins. The primary bacterial cells of *Photorhabdus* of (BA1, BAA1 and MH) cultured on nutrient bromthymol blue agar plates (NBTA) looked like broken glass with irregular margins. The dye was absorbed after 3 to 4 days after inoculation and incubation at 28°C. The colonies turned blue or dark purple. On the other hand, the secondary colonies had translucent flat margins with greater diameter (1-2 µm). Colonies of the primary form of BA1, BAA1 and MH absorbed the neutral red from the McConkey agar plates and turned red while the bacterial cells of the secondary form did not absorb the neutral red and remained off-white. McConkey agar is very convenient because on other media responses are obscured by the pigmentation of the strain (Babic *et al.* 2000). On this medium a poor growth of microorganisms is obtained.

In the case of the primary bacterial cells of *Xenorhabdus* (BA2) cultured on NBTA agar, the cells were large with an olive-blue color with uneven or irregular margins. Meanwhile, the secondary form cells were white. No light was emitted from both form variants. *Xenorhabdus* cells could not uptake the McConkey dye from the agar plates. Another remarkable difference between the *Photorhabdus* and *Xenorhabdus* colonies is that the colonies of the primary cells of *Photorhabdus* were sticky and gummy when removed from the agar plates while the *Xenorhabdus* cells were not.

Table 3 Detection of 16S rRNA gene in the four local isolates using four PCR-specific primers.

16S rRNA primers	Species and their local isolates			
	Xenorhabdus nematophila	*Photorhabdus luminescens*		
	BA2	BA1	BAA1	MH
TEM	+			
TRO		+	+	+
TYPE	+	+	+	+
PÜTZ	+	+	+	+

+= the presence of the 16S rRNA gene

Identification of 16S rRNA gene in the local bacterial isolates

Four specific primers (TEM, TRO, TYPE and PÜTZ) were designed to detect the 16S rRNA gene in the four local isolates. PCR amplification revealed the presence of amplified fragments characteristic of the four primers and the results showed the expected product size of the four primers with 355 bp.

Primer TRO displayed the 16S rRNA gene with the 355-bp fragment in the three isolates (BA1, BAA1 and MH) belonging to *P. luminescens*, while it was absent in the isolate BA2 belonging to *X. nematophila* (**Fig. 1; Table 3**). On the contrary, primer TEM showed the fragment of the 16S rRNA gene in the isolate BA2 and the gene was not detected in the three other isolates belonging to *P. luminescens* (**Fig. 1**). The two other primers (TYPE and PÜTZ) revealed the 355-bp fragment of the 16S rRNA gene in all four isolates.

Sequence analysis of PCR-amplified 16S rRNA of the local *Photorhabdus luminescens* isolates

A 355 bp nucleotide sequence of the partial 16S rRNA gene from BAA1 isolate (accession no. FJ755891) was aligned and compared in the GenBank using the BLAST program. A total of more than 50 16S ribosomal RNA gene partial sequences from different accessions of *P. luminescens*, subspecies, strains and isolates were identity with variable percentages (**Table 4**).

Blast alignment revealed several accession strains of five *P. luminescens* subspecies whose identity ranged from 82 to 76%, whereas a total of 12 strains of subspecies *akhurstii*, 14 of *laumondii*, 5 of *kayaii*, 4 of *luminescens* and 3 of *thracensis* were displayed as shown in **Table 4**. The highest level of identity (82%) was obtained in five strains of subspecies *kayaii*, followed by 77% identity in three strains (LN2, EG2 and IND) of *akhurstii* and one strain (IRA2) of subspecies *laumondii*. All the remaining strains had 76% identity. Moreover, Blast alignment showed identity with five *P. luminescens* isolates with 76% identity. Consequently, the 16S rRNA sequence of the *P. luminescens* isolate was compared to 16S rRNA sequences of *X.*

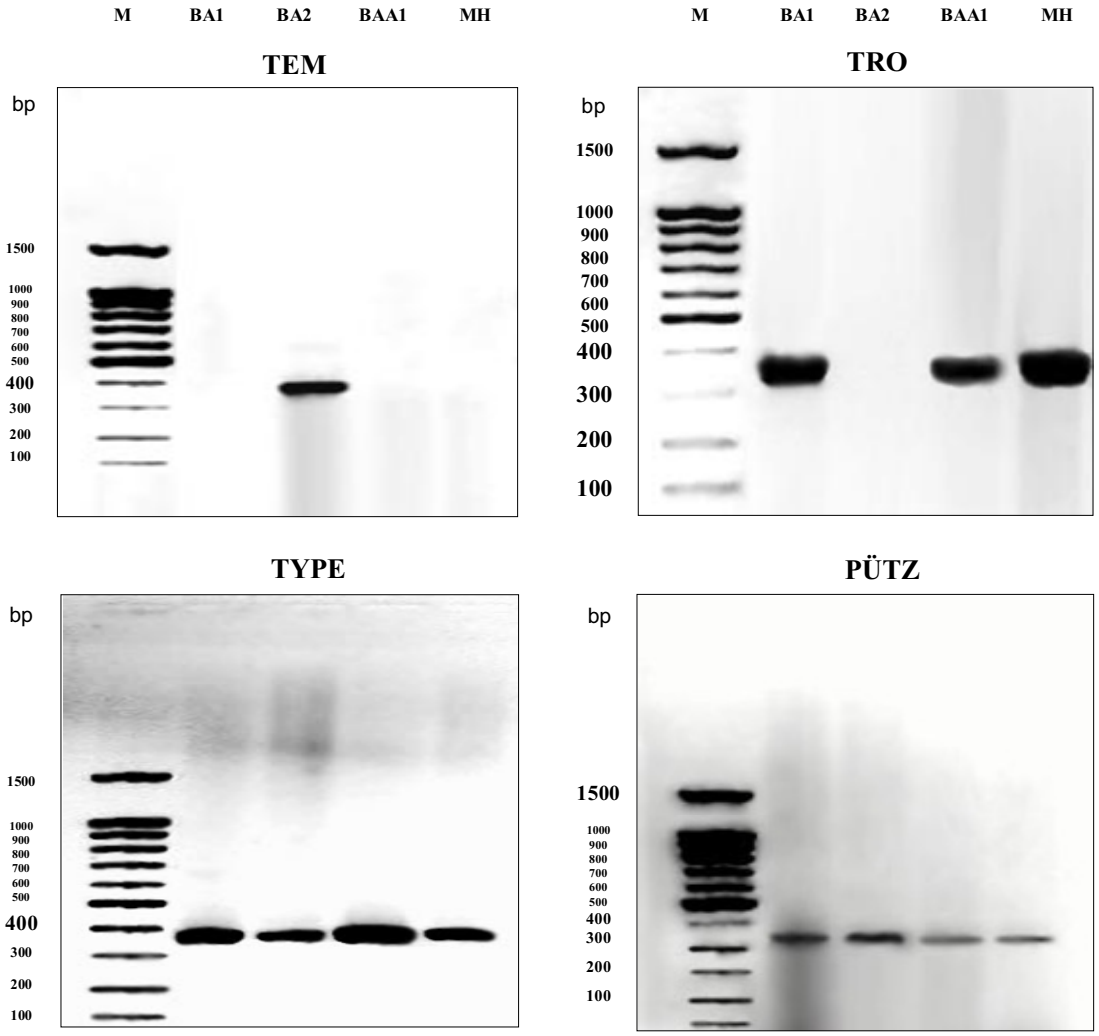

Fig. 1 PCR products of 16S rRNA gene using four designed primers in the four local isolates.

nematophila. Seven strains and two *X. nematophila* isolates were identity with 76% and one strain PDBC SCX3 had 82% identity, as shown in **Table 4**.

Sequence alignment (**Fig. 2**) of the 16S rRNA gene of the novel *P. luminescens* Egyptian isolates (FJ755891) compared with *P. luminescens* and *X. nematophila* GenBank isolates and strains revealed positional differences in nucleotide sequences between the novel local *Photorhabdus* isolate and numerous isolates and strains. Most of the total numbers of nucleotide positional differences ranged from 1 to 4 and the highest number (8) of positional differences was adenine (A) found in FJ755891 that changed to guanine (G) in all other GenBank isolates and strains as shown in **Table 5**. A single base change or a mixed base (more than one nucleotide determined at a single position) is considered as a new 16S type. The phylogenetic relationship between the local *P. luminescens* isolate (FJ755891) and all described *P. luminescens* and *X. nematophila* isolates and strains obtained from GenBank based on the 16S ribosomal RNA gene *Photorhabdus* species and subspecies is presented in **Fig. 3**. The dendrogram divided all named isolates and strains into three main discrete clusters, whereas the local isolate (FJ755891) formed a phylogenetically distinct cluster, separate from all other named isolates and species, while one of the two clusters contained all the GenBank isolates and the other contained all the GenBank subspecies strains.

DISCUSSION

The bacterial cells of the primary clone of the isolates BA1, BAA1 and MH cultured on NBTA were short thick rod-shaped cells with irregular margins and the colonies turned blue in color or dark purple (data not shown). On the other hand, the secondary form were long, thin cells whose colonies had translucent flat margins. Colonies of their primary form absorbed the neutral red from McConkey agar plates and turned red while the bacterial cells of the secondary form did not. The primary form also possessed two small ovoid inclusion bodies not presented in the secondary form. In isolate BA2, *X. nematophilus*, the colony shape and cell size were different from that of *P. luminescensce.* In the primary bacterial cells of *Xenorhabdus* of BA2 cultured on NBTA agar, the cells were large with olive-blue colours with uneven or irregular margins. Meanwhile, the secondary form cells were white. No light was been emitted from both form variants. It was noticed that the *Xenorhabdus* cells were not able to uptake the McConkey dye from the agar plates.

According to Akhurst and Boemare (1990), the secondary form of *Xenorhabdus* spp. is a stable colony variant that has lost its ability to produce pigments, antimicrobial agents, and secondary metabolites, cannot take up dye, does not have proteinaceous inclusion bodies and, in the case of *X. luminescens*, is not luminescent. The primary and secondary forms of most *Xenorhabdus* strains are equally pathogenic. The BA1 and BA2 variants on the other hand, differ significantly in their pathogenicity. This variation may be of importance for the pathogenicity of the nematode. When an infective nematode carries a less pathogenic bacterium in the intestine, the combination also will be less pathogenic (Gerritsen *et al*. 1992).

A striking feature of *P. luminescens* (and *Xenorhabdus* spp.) is phase variation, which affects a large number of

Table 4 16S rRNA gene sequence identity between the local *Photorhabdus luminescens* isolate sequence and GenBank *Photorhabdus luminescens* and *Xenorhabdus nematophila* sequences.

Subspecies	Strains	Accession	Total score	Coverage (%)	Identity (%)
Photorhabdus luminescens					
1. akhurstii	LN2	**AB355866**	172	85	77
	EG2	AY278644	167	″	″
	IND	AY278643	″	″	″
	1007-2	EF408911	171	95	76
	FRG0 16S	AJ007359	″	″	″
	0805-P5G	EU301784	″	″	″
	G 16S	AY822035	″	″	″
	R	AY822034	″	″	″
	W14	AY278642	″	″	″
	IS5	AY278645	167	″	″
	0813-124	DQ223040	161	85	″
	W0820-11	EF408910	156	″	″
2. laumondii	IRA2	**EU600196**	161	85	77
	Iran1	EU939526	171	95	76
	IRA10	EU600199	165	″	″
	IRA5	EU600198	″	″	″
	IRA1	EU600195	″	″	″
	FR42	EU190980	″	″	″
	Iran8	EU250473	″	″	″
	ARG	AY278650	″	″	″
	Az36	AY278649	″	″	″
	HP88	AY278648	″	″	″
	TUR2	AJ295162	″	″	″
	TT01	AJ007404	159	″	″
	SRK 2	EU513181	161	93	″
	IRA3	EU600197	156	85	″
3. kayaii	FR33	**EU930333**	165	53	82
	ITH-LA3	EU930334	159	″	″
	FR41	EU190979	″	″	″
	DSM 15197	AJ560632	″	″	″
	DSM 15198	AJ560631	″	″	″
4. luminescens	Hm	**AY278641**	176	95	76
	DSM 3368	X82248	171	″	″
	Hb	AY278640	″	″	″
	ATCC 29999	AY870658	″	″	″
5. thracensis	FR32	**EU930335**	176	″	″
	Iran3	EU122952	″	″	″
	DSM 15199T	AJ560634	″	″	″
Isolate (Aqaba)		EU214642	176	95	76
Isolate (Muaggar)		EU214641	″	″	″
Isolate (Balka-Arida 2)		EU214640	″	″	″
Isolate (Krf-Khal)		EU214639	″	″	″
Isolate (Kfr-Anja)		EU214638	″	″	″
Xenorhabdus nematophila					
	4	**FJ640983**	176	95%	76%
	CA01	DQ211705	″	″	″
	F1	AY521241	″	″	″
	Breton	DQ282116	″	″	″
	YL001	**EU124381**	171	″	″
	ES96	DQ211707	″	″	″
	BE06	DQ211704	″	″	″
	PL31	AY521242	″	″	″
	PDBC SCX3	**AY753196**	150	48%	82%
Isolate (Bdr-1)		**EU214636**	171	95%	76%
Isolate (Balka-Arida 1)		**EU214635**	″	″	″

membrane-bound, intra- and extracellular proteins and secondary metabolites (Akhurst *et al.* 1996). Phase I variants are involved in the symbiotic relationship with entomopathogenic nematodes and are isolated from the non-feeding infective stage nematodes and the body cavities of insects killed by these nematodes. No role in symbiosis has yet been determined for phase II, which is associated only with entomopathogenic nematodes under laboratory conditions. It is the potential for biological control of insect pests that drives most of the scientific research on these bacteria. *P. luminescens* was originally classified within the family *Enterobacteriaceae* as a species of the genus *Xenorhabdus* (Thomas and Poinar 1979).

The general phenotypic and molecular characterization discriminated congruently the three new Egyptian isolates (BA1, BAA1 and MH) of *P. luminescens* isolated from nematode *H. bacteriophora* and one isolate (BA2) of *X. nematophila* isolated from the nematode *S. carpocapsae*. PCR amplification of 16S rRNA using two of the four primers (TRO and TEM) designed within the 440 to 480 bp region under stringent annealing temperature at 65 and 68°C, respectively is considered a fast and reliable method to distinguish between the three isolates (BA1, BAA1 and MH) belonging to *P. luminescens* and isolate BA2 belonging to *X. nematophila*. However, the results of Ehlers and Niemann (1998), who initially synthesized the four primers, were partially in agreement with our results, whereas primer TRO based on strain DSM12191 (isolated from the nematode type strain *H. indica* strain LN2) was identified in *P. luminescens* of tropical origin isolated from *H. indica* and TEM based on the sequence of strain DSM12190 (isolated from North West European *H. megidis* strain HSH2) was identified all *P. luminescens* associated with *H. megidis* from North West Europe and two isolates from closely the related nematode strains from Ireland. Moreover, primer TYPE detected the 355 bp fragment only from the type strain ATCC 29999 of *P. luminescens*, while no products were obtained with strain HSH2 and by using PÜTZ primer, the fragment was not displayed in strain RS120.

Sequence alignment revealed 32 nucleotide positional differences between the novel local *Photorhabdus* isolate (FJ755891) and numerous isolates and strains of *P. luminescens* and *X. nematophila* based on the 16S rRNA gene similarity (**Fig. 2; Table 5**). Phylogenetic dendrogram analysis based on 16S rRNA gene sequences using distance, parsimony and maximum-likelihood criteria, showed that the local isolate (FJ755891) formed a phylogenetically distinct group, separate from all other named isolates and species (**Fig. 3**). Such obtained results evidently indicate a large diversity with unique characteristics of the local Egyptian isolates from all the other isolates and strains established around the world.

The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny) and more recently it has become important as a means to identify new isolates or an unknown bacterium to the genus or species level (Sacchi *et al.* 2002). Moreover, the gene was used for molecular typing isolates and strains belonging to *Xenorhabdus* and *Photorhabdus* spp. The phylogenetic tree displayed the relationships among species of *Xenorhabdus* (Lengyel *et al.* 2005) and *Xenorhabdus* are distinguished from *Photorhabdus* by the sequence TTCG at positions 208-211 of the 16S rRNA sequence (Boemare and Akhurst 1999). Sequence variation within the variable region of the 16S rRNA at position 440 to 480 allowed Ehlers and Niemann (1998) to synthesis specific PCR primers for the identification of groups within the species *P. luminescens*, symbionts of entomopathogenic nematodes of the genus *Heterorhabditis*.

Several reports support our findings and characterization of new isolates and strains of *Photorhabdus* and *Xenorhabdus* using 16S rRNA gene sequence. Tóth and Lakatos (2008) isolated one strain (3107T) from *Heterorhabditis downesi* and *Heterorhabditis megidis* that showed only moderate 16S rRNA gene sequence similarity to the type strains of all described *Photorhabdus* species and subspecies. Other isolates from nematodes of Turkish soil samples were characterized using 16S rRNA gene sequence similarities and metabolic properties and seven members of the genus *Photorhabdus* identified to the species level (Hazir *et al.* 2004). Liu *et al.* (2001) compared partial 16S rRNA sequences from the symbiotic bacteria of nematodes (*Heterorhabditis marelatus* and *Steinernema oregonense*) with sequence from previously described *Photorhabdus* and *Xenorhabdus* species. The 16S sequence from the new *Xenorhabdus* isolate appears very similar to, although not iden-

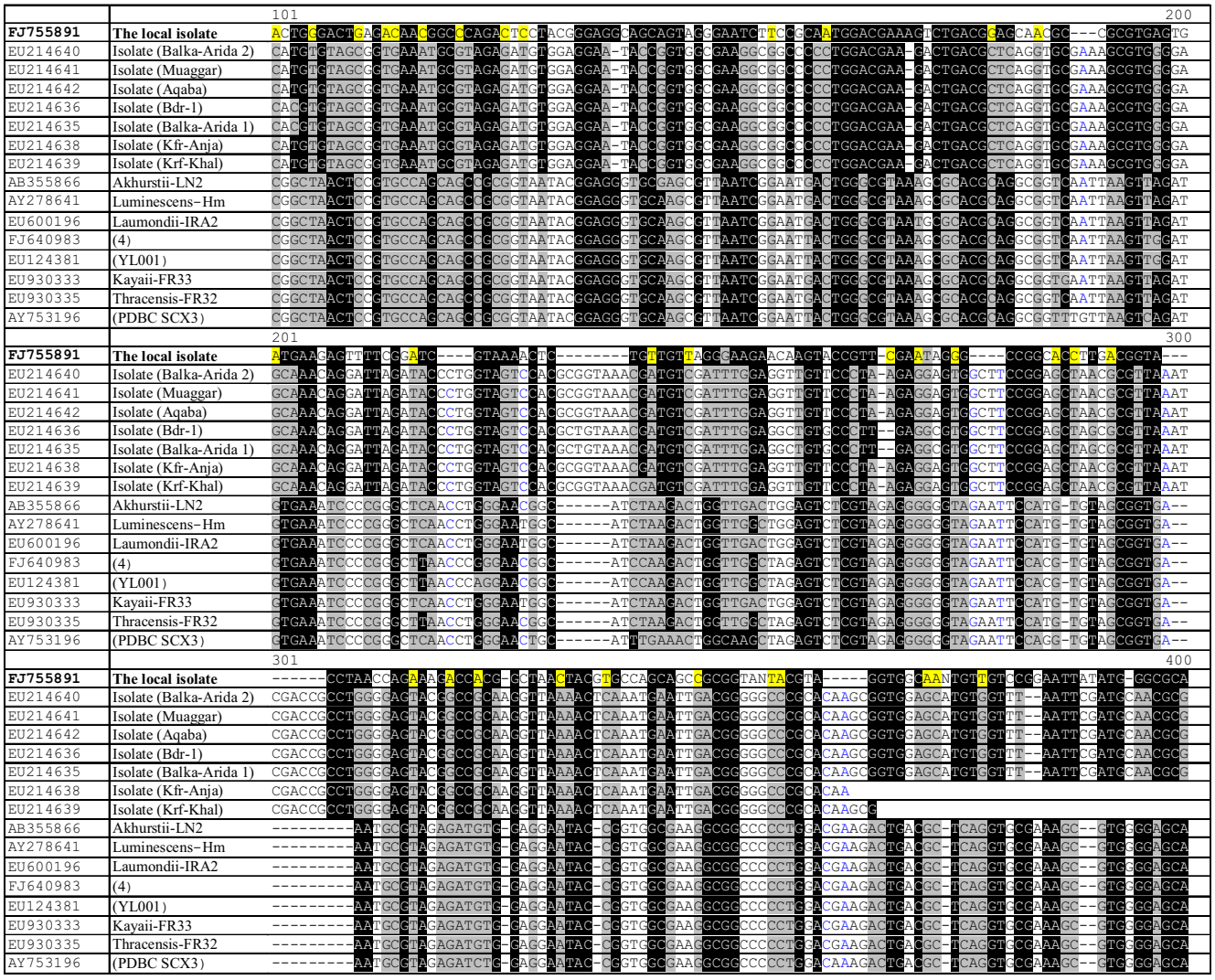

Fig. 2 Sequence alignment of 16S rRNA gene of the novel *Photorhabdus luminescens* Egyptian isolates (FJ755891) compared with *P. luminescens* and *Xenorhabdus nematophila* GenBank isolates and strains. Conserved nucleotides between FJ755891 and other sequences are boxed in black. Putative conserved between the different isolates with no identity with FJ755891 are boxed in grey. The yellow box referred to the identity of all accessions except FJ755891. Dashes correspond to gaps introduced to optimize the alignments. Blue letters indicate that nucleotides are similar in all accessions, except in the local isolate FJ755891.

Table 5 Positional differences in nucleotide sequences between the novel local *Photorhabdus* isolate and numerous isolates and strains based on the 16S rRNA gene similarity.

Existing in accession (FJ755891) as:	**A**	**A**	**A**		**C**	**C**	**C**		**G**	**G**		**T**	**T**	**T**
Changed in all other accessions to:	**C**	**G**	**T**		**A**	**G**	**T**		**T**	**C**		**A**	**C**	**G**
Changed at nucleotide positions:	101	184	113		117	114	128		105	110		242	246	155
	161	201	216		268	121	288		275	179			337	378
	373	271	316		332	126								
		286				347								
		292												
		320												
		323												
		372												
Total number of nucleotide positions = (32)	3	8	3	(14)	3	4	2	(9)	2	2	(4)	1	2	2 (5)

tical to, that of *X. bovienii*, the common symbiont of *S. feltiae*. The new *Photorhabdus* isolate appears to be very distinct from other known *Photorhabdus* species, although its closest affinities are with the *P. temperata* group. Tailliez *et al.* (2006) investigated the diversity of a collection of 76 *Xenorhabdus* strains, isolated from at least 27 species of *Steinernema* nematodes and collected in 32 countries. Their results of 16S rRNA sequences of the *Xenorhabdus* strains were highly conserved (similarity coefficient >95%), suggesting that the common ancestor of the genus probably emerged between 250 and 500 million years ago. Based on comparisons of the 16S rRNA gene sequences, they identified 13 groups and seven unique sequences. They classified new isolates into the *Xenorhabdus* species and described 10 novel *Xenorhabdus* species. Sergeant *et al.* (2006) characterized *Xenorhabdus* strains from nematodes isolated from UK soils by partial sequencing of the 16S rRNA gene, four housekeeping genes (*asd*, *ompR*, *recA*, and *serC*) and the flagellin gene (*fliC*). 16S rRNA sequences and the sequence types based on housekeeping genes were in agreement, with a few notable exceptions. In the search for novel *Xenorhabdus* strains in a recently described nematode species,

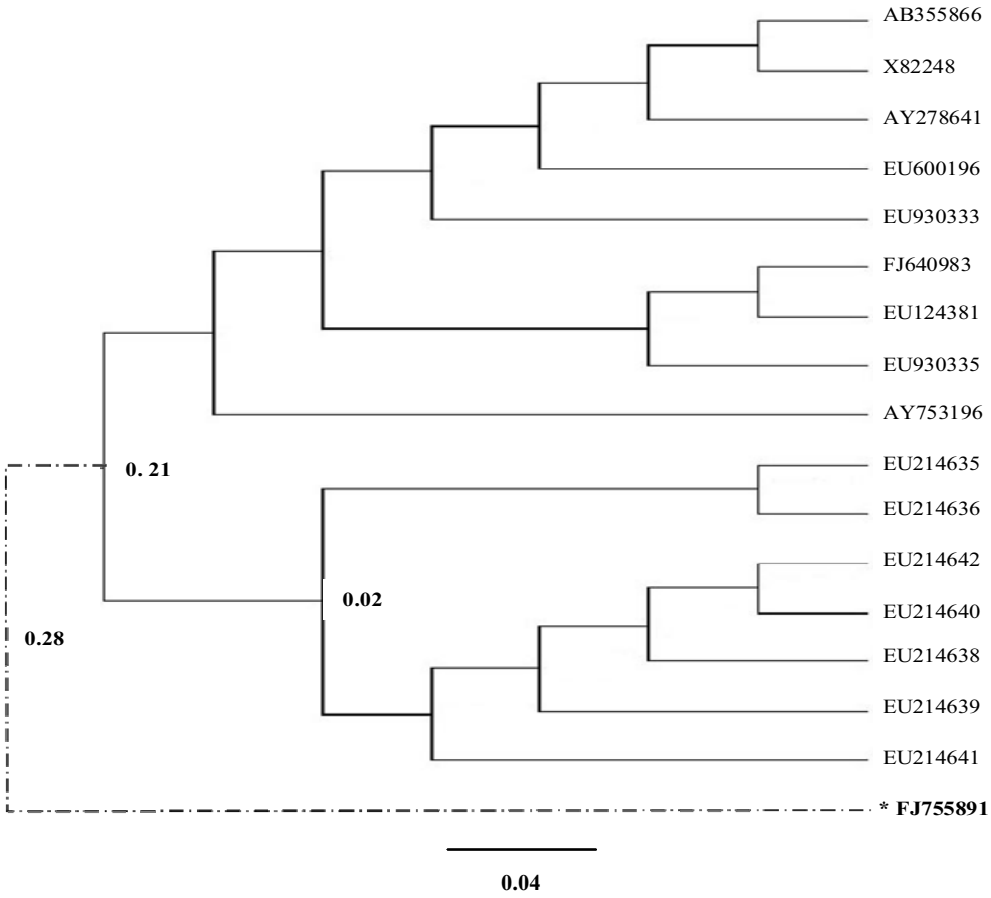

Fig. 3 Phylogenetic relationships between the local *P. luminescens* isolate (FJ755891) and both *P. luminescens* and *X. nematophila* isolates and strains obtained from GenBank, based on the 16S ribosomal RNA gene.

Steinernema thermophilum, three strains (DSM 17382, 17383 and 17384) were isolated from three independent isolation approaches from crushed mixture of infective juveniles. 16S rRNA gene sequence comparison indicated identity and the phylogenetic position pointed towards an individual taxon within the phylogenetic dendrogram of *Xenorhabdus* type strains (Somvanshi *et al.* 2006).

Consequently, the new Egyptian isolates of *P. luminescens* and *X. nematophila* that are naturally found in symbiotic associations with soil entomopathogenic nematodes may be strictly correlated with the geographical origins of the isolates and can be used as new commercial insecticidal bacteria in view of their potential for the development of novel biopesticides.

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