



Molecular and Phenotypic Characterization of Two Bacteria, *Photorhabdus luminescens* subsp. *akhurstii* HRM1 and HS1 Isolated from Two Entomopathogenic Nematodes, *Heterorhabditis indica* RM1 and *Heterorhabditis* sp. S1

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ABSTRACT

The present study was aimed at molecular and phenotypic characterization of two new bacterial strains isolated from entomopathogenic nematodes, *Heterorhabditis indica* RM1 from Alexandria, northern Egypt and *Heterorhabditis* sp. S1 from RasSidr, South Sinai, eastern Egypt. These entomopathogenic bacteria secrete lethal toxins that rapidly kill the insect host within 48h. They also produce various antibiotics that suppress infection by other microorganisms. Three complementary approaches including 16S rRNA gene sequencing, phenotypic and phylogenetic analysis by maximum likelihood method and chemical characterization, were used to identify these new isolates. Current investigations revealed that these two isolates had noteworthy differences, particularly in their antibiotic production spectrum from *P. luminescens* subsp. *akhurstii* 1S5; however, they maintain the major characters that define them from other species. A minor difference from regular *P. luminescens* subsp. *akhurstii* was obtained by 16S rRNA gene sequencing, which resulted in two new type strains: *Photorhabdus luminescens* subsp. *akhurstii* HRM1KC237382 and *Photorhabdus luminescens* subsp. *akhurstii* HS1KC237383.

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Authors' Contributions

HAE conceived the idea and performed all the experiment work. HAA participated in DNA isolation and sequencing. RAA and THA participated in bioinformatics analysis. SE provided lab facilities. AMA supervised the work and wrote the article with the help of HAE, HAA, RAA and THA.

Key words

Photorhabdus, molecular characterization, phenotypic characterization, antibiotic sensitivity test, entomopathogenic nematodes, *Heterorhabditis* spp.

INTRODUCTION

Two nematode genera *Steinernema* and *Heterorhabditis* are characterized by their mutualistic bacteria which have wide range application as insect pathogens in biological control of pests (Gaugler and Kaya, 1990). The infective juvenile nematodes penetrate into the insect host to inhabit inside the insect haemocoel (Forst and Clarke, 2002). Symbiotic bacteria are released from the nematode gut into the insect haemocoel, where they reproduce and convert the body contents of the insect host into a nutrient soup that is ideal for nematode nutrition, growth and reproduction. Finally, the nematodes reproduce and develop into new infective juveniles that are infected by the symbiotic bacteria to

form a new infective juvenile-bacteria symbiotic combination (Dowds and Peters, 2002; Forst and Clarke, 2002; Inman *et al.*, 2012). This combination kills the insect host within few hours, and thus, is considered one of the most effective biocontrol agent after the entomopathogenic bacteria (Ahmed 2012 and Ahmed *et al.*, 2014)

Gram-negative bacteria belonging to the genus *Photorhabdus* are associated with *Heterorhabditis* entomopathogenic nematodes (Boemare *et al.*, 1993). Phylogenetic evidence of the taxonomic heterogeneity of *Photorhabdus luminescens* was reported by Szállás *et al.* (1997), whereas, Fischer-Le Saux *et al.* (1999) described three species belonging to the genus *Photorhabdus* (*P. asymbiotica*, *P. luminescens* and *P. temperata*). Moreover, they described three subspecies of *P. luminescens* (*P. luminescens akhurstii*, *P. luminescens laumondii* and *P. luminescens luminescens*) and one (Illegitimate) subspecies of *P. temperata* (*P. temperata temperata*). Akhurst *et al.* (2004) described two subspecies of *P. asymbiotica* (*P. asymbiotica* subsp.

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australis and *P. asymbiotica* subsp. *asymbiotica*). Hariz *et al.* (2004) described two further subspecies of *P. luminescens* (*P. luminescens kayaii* and *P. luminescens thracensis*). Many new *Photorhabdus luminescens* strains have been isolated from South Africa (Ferreira *et al.*, 2014). Using basic phenotypic characterization to identify new bacterial isolates is the first step in identifying bacteria. However, a sequence analysis of 16S rRNA genes is useful for rapid identification of novel bacterial isolates. Although the advantages of this approach are clear, classification by rRNA gene sequencing alone is sometimes unsatisfying for several reasons, including a lack of resolution compared with quantitative hybridization and horizontal gene transfer. Therefore, a polyphasic approach that includes both molecular and phenotypic studies is recommended to obtain a more accurate classification at the molecular level. For example, for the genus *Xenorhabdus*, DNA-DNA hybridization, 16S rRNA gene sequences analysis in addition to phenotypic typing methods allowed the precise classification of isolates into different species (Tailliez *et al.*, 2006).

In Egypt, *Heterorhabditis* sp. S1 was isolated from warm coastal region of RasSidr and South Sinai, eastern Egypt, and *Heterorhabditis indica* RM1 was isolated from the cold coastal region of Alexandria, northern Egypt (El-Assal *et al.*, 2002). The aim of this study was to retype the *Photorhabdus* strains isolated from different geographical localities in Egypt using 16S rRNA in a trial to emphasize the coevolution extension of *Photorhabdus* distribution. Additionally, chemotaxonomic characterization, including an antibiotic sensitivity test was also done.

MATERIALS AND METHODS

Bacterial strains

The two strains of bacteria belonging to the genus *Photorhabdus* were isolated from the entomopathogenic nematodes, *Heterorhabditis* sp. S1 and *Heterorhabditis indica* RM1. The *Photorhabdus* genus of the S1 nematodes isolated from RasSidr, South Sinai was defined based on the pathogenic symptoms (the cadaver takes reddish color and gummy haemolymph) and their life cycle (10-12 days) in the infected *Galleria mellonella* larvae (Poinar, 1979). *Heterorhabditis indica* RM1 was isolated from Alexandria northern Egypt, as defined by El-Assal *et al.* (2002). The two nematode strains were propagated from 1998 up till now on the last instar larvae of *G. mellonella* according to Dutky *et al.* (1964) in the laboratory of Parasitology and Animal Diseases Department, National Research Centre, Giza, Egypt.

Isolation and culturing of bacterial strains

Grinding method was used to isolate the nematode symbiotic bacteria *Photorhabdus* spp. HRM1 and HS1 from *H. indica* RM1 and *Heterorhabditis* sp. S1, respectively according to Goetsch *et al.* (2006) with some modifications. Infective Juveniles (IJs) were surface sterilized by immersing in 1% sodium hypochlorite for 1 min and then washed three times with sterile distilled water. 50 µl of the nematodes suspension (about 100 IJs) was placed in a sterile 5 ml Petri dish, followed by 100 µl of sterile Luria-Bertani Miller (LB) broth (Difco). Sterile nematode (5-7 IJs) was picked and submerged in a sterile 100 µl of LB broth supplemented with 2.5% ampicillin. Using a sterile pestle, the nematodes were ground for 1 min on ice. Nutrient bromothymol blue agar (NBTA) media (2.3% "Difco" nutrient agar, 0.0025% "Merck" bromothymol blue, 0.004%, 2, 3, 5- triphenyltetrazolium) plates were incubated at 29°C, and single bacterial colonies were subcultured onto new NBTA plates until no contamination was detected. A single pure colony was subcultured into LB broth at 29°C for 24 h in a shaking incubator at 200 g.

Characterization of bacteria

Isolated *Photorhabdus* HRM1 and HS1 colonies were characterized according to morphology, pigmentation, shape and surface properties. These colonies were preserved by patch plating onto grid plates and frozen in 20% glycerol at -80°C. The plates were incubated at 30°C for 24h. For further characterization of the isolates, bacterial cultures were processed for Gram staining following the method described by Van der Hoeven *et al.* (2008).

DNA extraction and PCR amplification of 16S rRNA gene

DNA was extracted from 2 mL LB overnight culture of each bacterial isolate using the Edge BioSystems Bacterial Genomic DNA Purification Kit (Cat. #85171, Bio kits, USA) following the manufacture instructions. The universal bacterial primers forward: 5'-GTTTGATCCTGGCTCAG-3' and reverse: 5'CGGYTACCTTGTTACGACTT-3' were used in the PCR reaction.

A small amount of cells was scraped from the colony using a sterile pipette tip, suspended in 3 µl distilled PCR-grade water and boiled for 5 min. The boiled cells were added to 47 µl of a PCR mixture containing 2 µl forward and reverse primers, 2.5 mM MgCl₂, 200 µM dNTPs, 5 µl MgCl₂-free 10X Taq polymerase buffer and 1.25 U Taq DNA polymerase (Promega). PCR amplification of 16S rDNA was carried out with the following thermal cycle. Denaturing at 94°C for 30s, annealing at 55°C for 30s and extension

at 72°C for 1min for 30 cycles. PCR products were purified using the High Pure PCR Product Purification Kit (Cat.# 11732676001, Roche Life Science, USA) and analyzed on 0.7% agarose gels. Nucleotide sequence analysis of the purified PCR products was performed at DNA Sequencing and Genotyping Facility at the University of Chicago Cancer Research Center. A BLASTN search of the NCBI database was used for genus designation. The obtained sequences were aligned with CLUSTALW and compared with the sequences in Ribosomal Database Project II (RDP-II), using the Sequence Match function (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

Antibiotic sensitivity test

The symbiotic bacteria HRM1 and HS1 antibiotic sensitivity was investigated in comparison with *Xenorhabdus nematophilus* and *Xenorhabdus bovinii* that are used as standard positive control against the pathogenic bacteria *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus* sp., *Micrococcus luteus*, *Micrococcus* sp., *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Bacillus cereus* and *Paenibacillus* sp. which were kindly provided by Prof. Dr. Steven Forst, Biological Sciences Dept., University of Wisconsin, Milwaukee, Wisconsin, 53201 USA. Symbiotic bacteria under investigation were cultured overnight (O/N) in 2 ml LB selection media. The O/N bacteria were inoculated in fresh 5-10 ml of LB broth in 250 ml flask and incubated for 18 h in a shaking incubator at 29°C and 200 g. The cultured cells were calibrated on electro-photometer to 0.48 on the 600 nm to represent 4×10^7 cells/ml. Then, 6 µl of the bacterial cultures was spotted onto plates and incubated at 29°C for 24 h. On the second day, O/N cultures of the tested microorganisms were established in 5 ml of LB broth. On the third day 1 ml of chloroform was added to the lid of the plates and then the plates were inverted to expose the tested bacteria HRM1, HS1, *X. nematophilus* and *X. bovinii* to the fumes for 30 min. The plates were then aerated for 15 min and overlaid with 10 ml of 45°C sterile LB agar and inoculated with 1 ml of the tested pathogenic microorganisms. The plates were incubated at 37°C. After 24 and 48 h, the inhibition zones were measured in mm (Akhurst, 1982). This test was replicated three times.

Phenotypic characterization of bacterial strains

The two bacterial isolates HRM1 and HS1 subcultured in LB, MaConkey and NBTA media (Table I) were used for standard biochemical testing. Bioluminescence was observed on the LB agar plates after 24-48 h of growth. The phenotypic identities of the

strains were confirmed using morphological, biochemical and physiological characteristics, as defined by Boemare and Akhurst (1988). Phenotypic tests on agar plates (Ampicillin susceptibility, dye adsorption, colony pigmentation, antibiosis, gelatin hydrolysis, DNase, Oxidase, Catalase, Nitrate reduction, Glucose iron agar, Lactose fermentation, Starch hydrolysis, Urease and Coagulase) have been described previously (Akhurst *et al.*, 1996; Tailliez *et al.*, 2006).

Statistical analysis

SPSS version 14 computer program was used for all statistical analysis. One way ANOVA was calculated by *T*-test, when the results of *F*-test gave *P*-value less than 0.05. Duncan test was carried out to detect the significance between means of each parameter.

Table I. Biochemical characteristic of isolated *Photorhabdus luminescens* subsp. *akhurstii* type strains HRM1 and HS1. The experiment was repeated thrice.

Biochemical test	Type strain HRM1	Type strain HS1
Oxidase	-	-
Catalase	Low	Low
Nitrate reduction	-	-
Gelatin hydrolysis	-	-
Glucose iron agar	-	-
Lactose fermentation	-	-
Starch hydrolysis	-	-
DNAase	-	-
Urease	-	-
Coagulase	-	-
Pigment color	Dark yellow	Yellow
Phase one Luminance	High	Low

Bioinformatics analysis

Molecular phylogenetic analysis was carried out by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura and Nei (1993) model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 6 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1025 positions in the final dataset. Evolutionary analyses were

conducted in MEGA5 (Tamura *et al.*, 2011). The partial PCR amplification of 16S rRNA gene of strains *Photorhabdus* HRM1 and HS1 were approximately 1550 bp amplicons and the consensus sequences of the *Photorhabdus* spp. were submitted to the GenBank with accession numbers KC237382 and KC237383, respectively.

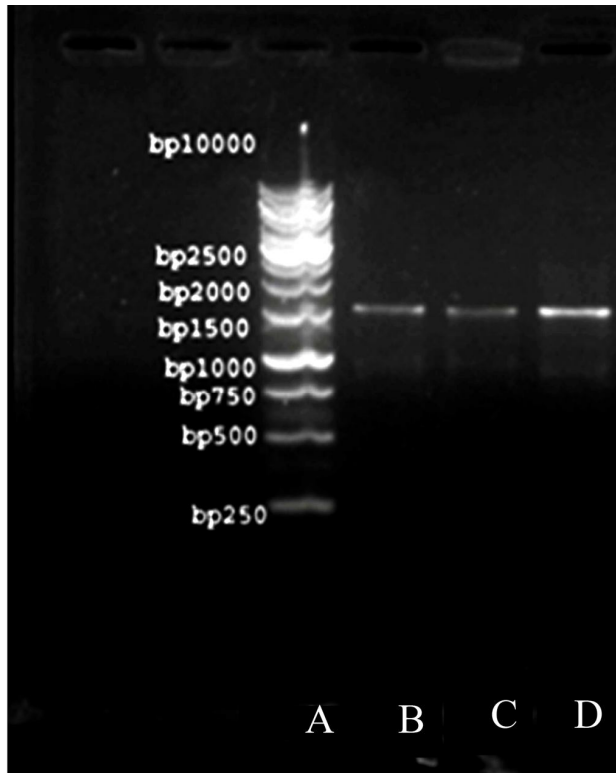


Fig. 1. Agarose gel (0.7%) electrophoresis of PCR amplified products of 16S rRNA genes of the bacterial isolates. A: ladder, B: *Photorhabdus luminescens* subsp. *lamondii* (HP88), C: *Photorhabdus luminescens* subsp. *akhurstii* HRM1 (RM1), D: *Photorhabdus luminescens* subsp. *akhurstii* HS1 (S1).

RESULTS

Retyping of *Photorhabdus* bacterial isolates

Figure 1 shows PCR products of 16S rRNA gene of HRM1 or HS1 isolates and the standard *P. luminescens lamondii* HP88. The sequences identified in this study were compared with the partial 16S rRNA gene sequences of the *Photorhabdus* isolates that are available from Gen Bank databases. The obtained results of Maximum Likelihood method revealed that, species under investigation, HRM1 and HS1 were almost

identical to *P. luminescens akhurstii* 1S5 AY378645 which are published by Fischer-Le Saux *et al.* (1999). The identity percentage was 100% and 99.098% for HRM1 KC237382 and HS1 KC237383, respectively (Fig. 2). The tree with the highest log likelihood (1490.4706) is shown in Figure 1. On the other hand, the three other evolutionary related species *P. luminescens akhurstii* W14, 0805-P5G and 276745.1 that are obtained from the Genebank are located in another distant branch with similarity of about 99.098, 99.098 and 99.096%, respectively. These sequences were compared with standard *P. luminescens lamondii* HP88.

Phenotypic analysis

Microscopic and biochemical observations revealed that, the two studied *Photorhabdus* types are Gram-negative, rod-shaped bacteria that are motile with peritrichous flagella. Phase I adsorbed dyes from MacConkey agar and NBTa medium. HRM1 colony was highly bioluminescent and gave dark yellow color on LB media, whereas, HS1 colony was less bioluminescent and gave light yellow color on LB media. However, both strains were ampicillin resistance and produced catalase. Also, they gave negative reactions for the oxidase activity, nitrate reduction to nitrite, DNase, urease production, coagulase, gelatin hydrolysis and hydrogen sulfide production tests. The carbohydrate hydrolysis as a carbon source showed an inability to hydrolyze lactose and starch (Table I).

Antibiotic sensitivity

Inhibition zones were measured (mm) for the primary form of HRM1 and HS1 against a panel of pathogenic microorganisms, including *Staphylococcus* sp., *S. epidermidis*, *S. aureus*, *M. luteus*, *Paenibacillus* sp., *E. coli*, *Micrococcus* sp., *S. typhimurium*, *K. pneumoniae* and *B. cereus* (Table II). The statistical analysis of inhibition zones indicated significant variations between the tested bacterial groups. Whereas, inhibition zone of *Staphylococcus* sp. (14.3, 12.7 mm) revealed high significant sensitivity, followed by *S. epidermidis* (12.3, 10.7 mm) against the studied bacteria HRM1 and HS1, respectively. No significant differences between inhibition zones of *M. luteus* (10.7, 12.7 mm) and *S. aureus* (9.7, 10.7 mm) against HRM1 and HS1, respectively were obtained. *S. aureus* was moderate sensitive, with inhibition zone towards HRM1 and HS1, respectively. *Micrococcus* sp., *S. typhimurium*, *K. pneumoniae* and *B. cereus* showed resistance against HRM1 and HS1 (Table II). Also, no significant variations between antibiotic activity of HRM1 and HS1 with all pathogenic bacteria were obtained.

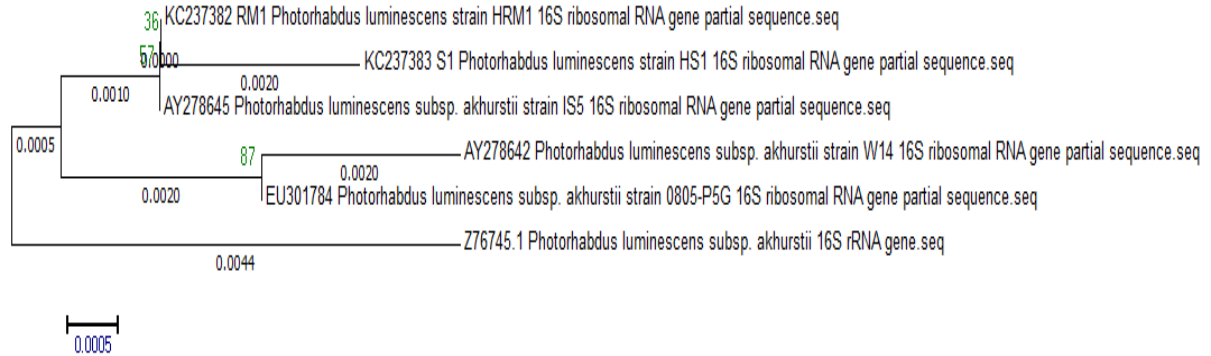


Fig. 2. Molecular phylogenetic analysis of *Photorhabdus luminescens* subsp. *akhurstii* HRM1 KC237382 and HS1KC237383 obtained by Maximum Likelihood method.

Table II. Inhibition zone diameters (mm) induced by *Photorhabdus luminescens* subsp. *akhurstii* type strains HRM1 and HS1 against variable bacterial pathogens.

Tested organism	Type strain HRM1		Type strain HS1	
	Colony zone (colz)	Inhibition zone (inz)	Colony zone (colz)	Inhibition zone (inz)
<i>Staphylococcus epidermidis</i>	5.3±0.33ab	12.3±0.33c*	4.7±0.33a	10.7±0.33b
<i>Staphylococcus aureus</i>	5±0.57a	9.7±0.33b	5.7±0.33abc	10.7±0.33b
<i>Staphylococcus</i> sp.	5±0.57a	14.3±0.33d	6±0.00bcd	12.7±0.33c
<i>Micrococcus luteus</i>	5.7±0.33abc	10.7±0.33b	5.7±0.33abc	12.7±0.33c
<i>Paenibacillus</i> sp.	5.3±0.33ab	7.7±0.33a	5.3±0.33ab	7.7±0.33a
<i>Escherichia coli</i>	6.7±0.33bc	7.7±0.33a	6.3±0.33bcs	7.7±0.33a
<i>Micrococcus</i> sp.	5.7±0.33abc	Faint	5.7±0.33abc	faint
<i>Salmonella typhimurium</i>	6.3±0.33abc	-	6.3±0.67bcd	-
<i>Klebsiella pneumonia</i>	7±0.00c	-	7±0.00d	-
<i>Bacillus cereus</i>	6±0.57abc	-	6.7±0.33cd	-
<i>F</i> value	2.844	62.567	3.798	45.600
<i>P</i> value		0.025	0.00	0.006

Inhibition and colony zone diameters are presented as (mean ± standard errors) of triplicate measurements 48h post-application. Differences between the two studied bacterial antibiotics (HRM1 and HS1) are analyzed for significance using T- test. Differences between bacterial groups are analyzed for significance using one-way analysis of variance. *F*- value with *P* < 0.05 according to Duncan's multiple range tests are significant. Columns with different letters (a, b, c, d) are significant according to Duncan test, while means of the same letters indicate non-significant difference.

These results were compared with the positive controls of *X. nematophilus* and *X. bovinii*. Where their antibiotic activity inhibited the growth of *S. epidermidis*, *S. aureus*, *Staphylococcus* sp., *Micrococcus* sp. and *M. luteus* with the exception of *S. typhimurium*.

DISCUSSION

Photorhabdus has been described as bioluminescent and facultative anaerobic insect pathogenic strains that are symbiont of entomopathogenic nematodes of the family Heterorhabditidae (Boemare *et al.*, 1993). The present study introduced two new types of

P. luminescens subsp. *akhurstii* (HRM1 and HS1) that were isolated from soil of two different locations in Egypt. The results of the partial gene sequencing of 16S rRNA indicated that these strains were identical strains of *Photorhabdus luminescens* subsp. *akhurstii* strains depicted in the GenBank data base according to the work of Fischer-Le Saux *et al.* (1999). Similarly, Hariz *et al.* (2004) used a combination of 16S rRNA gene sequences and phenotypic traits in the identification of *P. luminescens* sp. *Kayaii* and *P. luminescens* sp. *thracensis*. Moreover, Lengyel *et al.* (2005) and Somvanshi *et al.* (2006) identified the other genus of the symbiotic bacteria *X. budapestensis*, *X. ehlersii*, *X. indica*, *X. innexi*

and *X. szentirmaii*. Recently, Orozco *et al.* (2013) used 16S rDNA and a selection of protein coding genes to characterize new species and subspecies of the bacterial symbiont of *Heterorhabditis sonorensis*, an entomopathogenic nematode species from the Sonoran desert in Arizona, USA.

Phenotypic characteristics of the studied bacterial strains HRM1 and HS1 have also been confirmed by studies of Fischer-Le Saux *et al.* (1999).

The primary forms of *Xenorhabdus* and *Photorhabdus* spp. produced effective antibiotics of different strength against gram-positive and gram-negative micro-organisms, as well as yeasts and plant pathogenic fungi (Fodor *et al.*, 2010; Wang *et al.*, 2011). The cell free primary forms of both new types of *Photorhabdus* HRM1 and HS1 produced a clear inhibition zones against *S. epidermidis*, *S. aureus*, *Staphylococcus* sp., *M. luteus*, *Paenibacillus* sp. and *E. coli*. These results are confirmed with the finding of Fodor *et al.* (2010) whereas, they indicated that the symbiotic bacteria *X. budapestensis*, *X. szentirmaii*, *X. innexi*, *X. ehlersii*, *X. nematophila*, *X. bovienii* and *X. cabanillassii* produce effective antibiotics against *E. coli*, *K. pneumoniae*, *S. aureus*. Conversely, the symbiotic bacteria HRM1 and HS1 produce faint inhibition zones towards *Micrococcus* sp. and no activity was obtained towards *S. typhimurium*, *K. pneumoniae* and *B. cereus*. Moreover, the bioassay for *Xenorhabdus* spp. antibiotic activity based on the overlay test exhibited significant positive activity against three groups of mastitis isolates from dairy cows (Furgani *et al.*, 2008). Considerable variability of antibiotic activity of the two studied *Photorhabdus* HRM1, HS1 and the positive controls *X. nematophilus*; *X. bovienii* are detected. These results revealed high degree of specificity imposed on the symbiotic relationship between the bacteria and nematode. This specificity is generally more restrictive for the *Heterorhabditis-Photorhabdus* pair where only one species of nematode retains one species of bacteria. However, *Steinernema-Xenorhabdus* symbiosis reflects less restriction where several different nematode species can retain and grow on the same *Xenorhabdus* species (Akhurst and Boemare, 1990). Furthermore, their efficacy compared with other strains remains to be investigated. Native species of entomopathogenic nematodes together with their symbiotic bacteria that are adapted to local environmental and climatic conditions are especially good candidates for use as biological control agents. The objective of the present study was to isolate EPNs symbiotic bacteria, derived from *P. luminescens* subsp. *akhurstii* associated with entomopathogenic nematodes *H. indica* RM1 and *Heterorhabditis* sp. HS1 from Egyptian soil. This study

may open new insight for recording the topographical extension of genus *Photorhabdus* in Egyptian soil and many other soils in Saudi Arabia and Africa for future biological control programs and to identify them based on 16S rRNA gene sequences.

CONCLUSION

Toxin purification from these two types of bacteria is now underway, which may be helpful in the battle against mosquito-borne diseases.

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