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Characterization of symbiotic and associated bacteria from entomopathogenic nematode *Heterorhabditis* sp. (nematode: Heterorhabditidae) isolated from India

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Abstract

Background: Entomopathogenic nematodes (EPNs) harboring symbiotic bacteria are one of the safest alternatives to the chemical insecticides for the control of various insect pests. Infective juveniles of EPNs locate a target insect, enter through the openings, and reach the hemocoel, where they release the symbiotic bacteria and the target gets killed by the virulence factors of the bacteria. *Photorhabdus* with *Heterorhabditis* spp. are well documented; little is known about the associated bacteria.

Main body: In this study, we explored the presence of symbiotic and associated bacteria from *Heterorhabditis* sp. (IISR-EPN 09) and characterized by phenotypic, biochemical, and molecular approaches. Six bacterial isolates, belonging to four different genera, were recovered and identified as follows: *Photorhabdus luminescens*, one each strain of *Providencia vermicola*, *Pseudomonas entomophila*, *Alcaligenes aquatilis*, and two strains of *Alcaligenes faecalis* based on the phenotypic, biochemical criteria and the sequencing of 16S rRNA gene.

Conclusion: *P. luminescens* is symbiotically associated with *Heterorhabditis* sp. (IISR-EPN 09), whereas *P. vermicola*, *P. entomophila*, *A. aquatilis*, and *A. faecalis* are the associated bacteria. Further studies are needed to determine the exact role of the bacterial associates with the *Heterorhabditis* sp.

Keywords: *Heterorhabditis* sp., *Photorhabdus luminescens*, 16S rRNA gene sequencing, biological control

Background

Entomopathogenic nematodes (EPNs) are obligate pathogens of insects in nature. The infective juveniles (IJs) of EPNs harbor the bacterial symbionts in their gut, and after entry into the target insect, the bacterial symbionts are released into the insect hemocoel. The IJs feed on the insect hemolymph and multiply enormously; the host dies within 24–72 h due to synergistic action of the nematode and the symbiotic bacteria (Ali et al. 2005).

Both the nematode and the bacteria go on multiplying on the insect cadaver until a new generation of IJs emerges, which further carry the symbionts to another target host and the infection cycle continues (Kaya and Gaugler 1993). EPNs from the *Heterorhabditis* and *Steinernema* genera are symbiotically associated with bacteria from genus *Photorhabdus* and *Xenorhabdus*, respectively. *Photorhabdus* is found throughout the whole intestine whereas *Xenorhabdus* is sheltered inside a special vesicle in an interior part of intestine (Bird and Akhurst 1983). In the absence of their symbionts, nematodes cannot develop beyond the J1 stage. It is due to the combined effect of both the symbionts that the

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insect pest is killed in a shorter period of time. EPNs due to their wide host range, ease at handling, short life cycle, amenability to large scale production, and eco-friendly nature have a great potential to be used as biological control agents against a wide range of insect pests (Ali et al. 2005; Dillman and Sternberg 2012).

The ease of their production and exemption from registration requirements are the two big reasons for commercial developments of EPNs. They are widely distributed in different soil habitats and are diverse with respect to the conditions needed for their survival, infectivity potential, mode of replication, and host range (Ali et al. 2005). Other major bacterial agents used in biological control of insect pests are *Bacillus thuringiensis* and *B. sphaericus*; these bacteria have been used in controlling insect pests belonging to the orders Diptera, Lepidoptera, and Coleoptera (Vega and Kaya 2012). Invariably, this group of bacteria is only effective after a susceptible insect has ingested their toxin (Pigott and Ellar 2007). This type of “passive infection” apparently limits the degree of efficiency of these bacteria to control the pests. However, the symbiotic bacteria of EPNs (*Photorhabdus* and *Xenorhabdus* spp.) are capable of causing “active infection,” which have a wide host range and are introduced into an insect host by their EPNs symbiont. Their pathogenicity is based on their ability to multiply within the hemocoel, elaborate toxic substances, and cause septicemia. The nematode bacteria complex is presently used for controlling a variety of pests in agriculture (Ruiu 2015).

Previously, some exploratory studies have been carried out to isolate EPNs from spice agro-ecosystems and to evaluate them against insect pests (Pervez et al. 2014a). However, knowledge of their symbiotic and associated bacteria is lacking. Although the interaction of *Photorhabdus* and *Xenorhabdus* with *Heterorhabditis* spp. and *Steinernema* spp., respectively, are well documented, little is known about the associated bacteria. However, it is increasingly apparent that other bacteria co-inhabit or colonize the insect cadaver and the nematode in addition to the typical symbionts.

Hence, present study aimed to characterize the symbiotic and associated bacteria from *Heterorhabditis* sp. (IISR-EPN 09), using polyphasic approach.

Main text

Materials and methods

Entomopathogenic nematode and insect sources

Infective juveniles (IJs) of *Heterorhabditis* sp. (IISR-EPN 09) were cultured as per the procedure described by Woodring and Kaya (1988). Freshly harvested IJs were surface sterilized with 0.01% hyamine solution and stored in distilled water in tissue culture flasks for further study. Greater wax moth *Galleria mellonella* (L.)

larvae were reared on artificial diet as per the procedure described by David and Kurup (1988). The larvae were sorted out visually and those of the same size (18 mm) were taken for the study.

Isolation of bacteria

The symbiotic and the associated bacteria from *Heterorhabditis* sp. (IISR-EPN 09) were isolated by the following 3 methods; in all the 3 methods, the freshly harvested IJs of *Heterorhabditis* sp. (IISR-EPN 09) were collected and sterilized in 0.01% hyamine solution for 2 min and washed thrice in sterile distilled water. The last wash was plated as control to check the efficacy of the surface sterilization procedure.

Maceration method The IJs were punctured by using a sterile platinum wire (Akhurst and Boemare 1988). The suspension containing the body contents was diluted and plated on nutrient agar supplemented with 25 mg l⁻¹ bromothymol blue and 40 mg l⁻¹ 2,3,5-triphenyltetrazolium chloride (NBTA) indicator plates (Akhurst 1980). Bacterial colonies were picked and purified subsequently on nutrient agar plates maintained at 28 °C for 72 h. The stock cultures were maintained at -80 °C.

Incubation method Surface sterilized IJs were directly inoculated into microfuge tubes containing 1 ml nutrient broth. The tubes were incubated at 28 °C for 72 h. After observing turbidity in the medium, a loop full was streaked on to NBTA plates and purified subsequently on nutrient agar.

Hemolymph drop method *G. mellonella* larvae infected with *Heterorhabditis* sp. (IISR-EPN 09) were surface sterilized in 70% ethanol and dissected by using a sterile scalpel under a laminar air flow. A drop of hemolymph was collected from the infected larvae, streaked onto NBTA medium, and incubated at 28 °C for 72 h (Park et al. 1999). Following incubation, the colonies were picked and subsequently purified on nutrient agar.

Phenotypic characterization

The growth characteristics of each isolate were studied at 28 °C and pH 7.0. The colony characteristics like size, consistency, pigmentation, color, form, and margins were recorded on nutrient agar supplemented with 2,3,5-triphenyltetrazolium chloride (TTC). The isolates were further subjected to Gram's reaction as described previously (Smibert and Krieg 1994).

Biochemical characterization

The biochemical tests to characterize the bacterial isolates viz., catalase, oxidase, indole, methyl red, Voges-Proskauer, citrate utilization, urease, triple sugar iron (TSI), esculin

hydrolysis, lipase production, carbohydrate fermentation test using sugars glucose, lactose, sucrose, maltose, and mannitol were carried out using standard protocols.

Amplification and sequencing of 16S rRNA gene

The genomic DNA from the bacterial isolates was extracted from 10 ml of overnight grown cultures and purified manually, using phenol chloroform isoamyl alcohol method. The cells were lysed by using lysozyme and proteinase K as described by Sambrook and Russell (2001). The quality and quantity of the genomic DNA was assessed by agarose gel electrophoresis and spectrophotometrically by using Biophotometer (Eppendorf, Germany). One hundred nanograms of the purified DNA from each isolate was used for the amplification of 16S rRNA gene in a 50- μ l reaction volume. The universal primers used for the amplification were 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCCGCA-3') (Zhou et al. 1997). The mixture further contained 1X Taq buffer, MgCl₂ at 1.5 mM, dNTP mix at 200 mM, sense and antisense primers at 10 pM each, and 1 U of Taq DNA polymerase. The conditions for the amplification were as: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 60 s, annealing at 52 °C 30 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 5 min. The amplified products were agarose electrophoresed and visualized under UV, followed by gel purification of the desired amplicon using GeneJET gel extraction kit (Fermentas, USA) to remove the unutilized dNTPs and primers. The purified amplicons were sequenced using the same primers that were used for the amplification with fluorescent terminators (Big Dye, Applied Bio systems; Eurofins Genomics India). The identity of the sequences acquired was ascertained by performing BLAST (Altschul et al. 1990).

Phylogenetic analysis

The evolutionary relationship of the 16S rRNA gene sequences was inferred, using the neighbor-joining method (Saitou and Nei 1987) in MEGA v 6.0 (Tamura et al. 2013). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2013) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated.

Results and discussion

Isolation of the bacteria

The absence of growth in the plates inoculated with the last wash after surface sterilization confirmed that the

surface sterilization procedure using hyamine (0.01%) solution for 2 min was effective. Bacteria were successfully isolated on NBTa (Supplementary Fig. 1) from the gut of EPNs, using all the 3 methods, but rate of recovery varied among the methods. A total of 6 isolates showing distinct and characteristic colony characteristics were taken for further studies (Table 1).

Phenotypic characterization

The colony characteristics like size, shape, elevation, texture, and pigmentation of each isolate was studied and recorded after 24 h of growth at 28 °C (Table 2). All the 6 isolates were found to be rod shaped, motile, and gave negative reaction to Gram's stain (Table 2; Fig. 1). Among the bacterial isolates, 5 had regular colony shape and all 6 displayed smooth texture. All the isolated bacteria showed red or dark red color colonies with off white margin on TTC (Table 2).

Biochemical characterization

The results of the biochemical tests performed for all the 6 bacterial isolates recovered from *Heterorhabditis* sp. (IISR-EPN 09) are given in (Table 3 and Fig. 2). All the isolates were positive for the production of catalase, ability to utilize glucose, and ability to utilize citrate as sole source of carbon, whereas the isolates were unable to utilize lactose, produce urease, and hydrolyze esculin. All the 6 isolates, except IISR-EPN BC 06, were able to ferment maltose and 2 out of 6 isolates (IISR-EPN BC 06 and IISR-EPN BC 10) were unable to ferment mannitol (Table 3).

Molecular characterization

Single discrete bands of approximately 1500 bp in size corresponding to the 16S rRNA gene were successfully obtained in all the six isolates. The amplicons were sequenced, and the sequences obtained were trimmed and subjected to BLAST. Based on the BLAST analysis, the isolates showed identity with different species of gram-negative bacteria belonging to family Enterobacteriaceae (Table 1). The use of 16S rRNA sequencing for bacterial identification is a reliable method and when used in combination with other classical methods (morphological and biochemical) its accuracy increases further. The various reasons 16S rRNA are used for reliable identification of prokaryotes are as follows: its widespread presence in all bacteria and Archaea, it has both conserved and variable domains (helping in tracing the evolutionary changes over a period of time), and its length (\approx 1500 bp) is ideal for sequencing and analysis (Patel 2001).

Of the 3 methods used for isolation of symbiotic bacteria, 2 methods yielded *Alcaligenes faecalis* and one led to the recovery of *Photorhabdus luminescens*, *Alcaligenes*

Table 1 List of associated and symbiotic bacterial isolates recovered

Methods	No. of isolates	Name of the isolate	Bacterial species
Maceration	01	IISR-EPN BC 06	<i>Providencia vermicola</i>
Hemolymph drop	03	IISR-EPN BC 07	<i>Alcaligenes aquatilis</i>
		IISR-EPN BC 08	<i>Alcaligenes faecalis</i>
		IISR-EPN BC 12	<i>Photorhabdus luminescens</i>
Incubation	02	IISR-EPN BC 10	<i>Pseudomonas entomophila</i>
		IISR-EPN BC 11	<i>Alcaligenes faecalis</i>

aquatilis, *Pseudomonas entomophila*, and *Providencia vermicola* (Table 1). The actual symbiont *P. luminescens* was obtained only by hemolymph drop method; however, it would be difficult to establish the supremacy of any of the 3 methods owing to the small sample size taken and the limited number of isolates recovered. The description of the isolates identified is as the following.

***Providencia vermicola* (IISR-EPN-BC 06)**

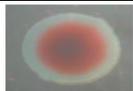
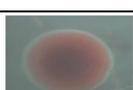
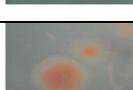
Providencia vermicola are gram-negative rods. Colonies are circular, shining, slimy, convex, and opaque with a brownish center and hyaline periphery. IISR-EPN-BC 06 showed 99% similarity with *P. vermicola* strain 6G (accession no. KC775772), *P. vermicola* strain FFA6 (accession no. JN092794), and *P. vermicola* strain CGS6 (accession no. KF886276). The optimal tree with the sum of branch length was 18.19960570. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the

phylogenetic tree. The analysis involved 13 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1170 positions in the final dataset (Fig. 3 a).

***Alcaligenes aquatilis* (IISR-EPN BC 07)**

Alcaligenes aquatilis is a gram-negative, rod-shaped, motile, non-nitrate reducing, oxidase positive, catalase positive, alpha hemolytic, and citrate-positive obligate aerobe that is commonly found in the environment. *A. aquatilis* belongs to phylum Proteobacteria, class Betaproteobacteria, order Burkholderiales, and family Alcaligenaceae. IISR-EPN BC 07 shows 97% similarity with *A. aquatilis* strain LMG22996 (accession no. NR104977). The optimal tree with the sum of branch length was 12.36487468. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The

Table 2 Phenotypic characteristics of associated and symbiotic bacteria

Isolates	Cell shape	Colony morphology						Grams reaction	Motility
		Size	Shape	Elevation	Texture	Color	Colony character on TTC		
IISR-EPN BC 06	Short rods	Small	Round	Raised	Smooth	Light red		-	+
IISR-EPN BC 07	Long rods	Large	Round	Raised	Smooth	Red with off white margin		-	+
IISR-EPN BC 08	Long rods	Medium	Round	Raised	Smooth	Dark red		-	+
IISR-EPN BC 10	Short rods	Small	Round	Flat	Smooth	Light red		-	+
IISR-EPN BC 11	Short rods	Small	Round	Raised	Smooth	Dark Red		-	+
IISR-EPN BC 12	Short rods	Medium	Irregular	Raised	Smooth	Red		-	+

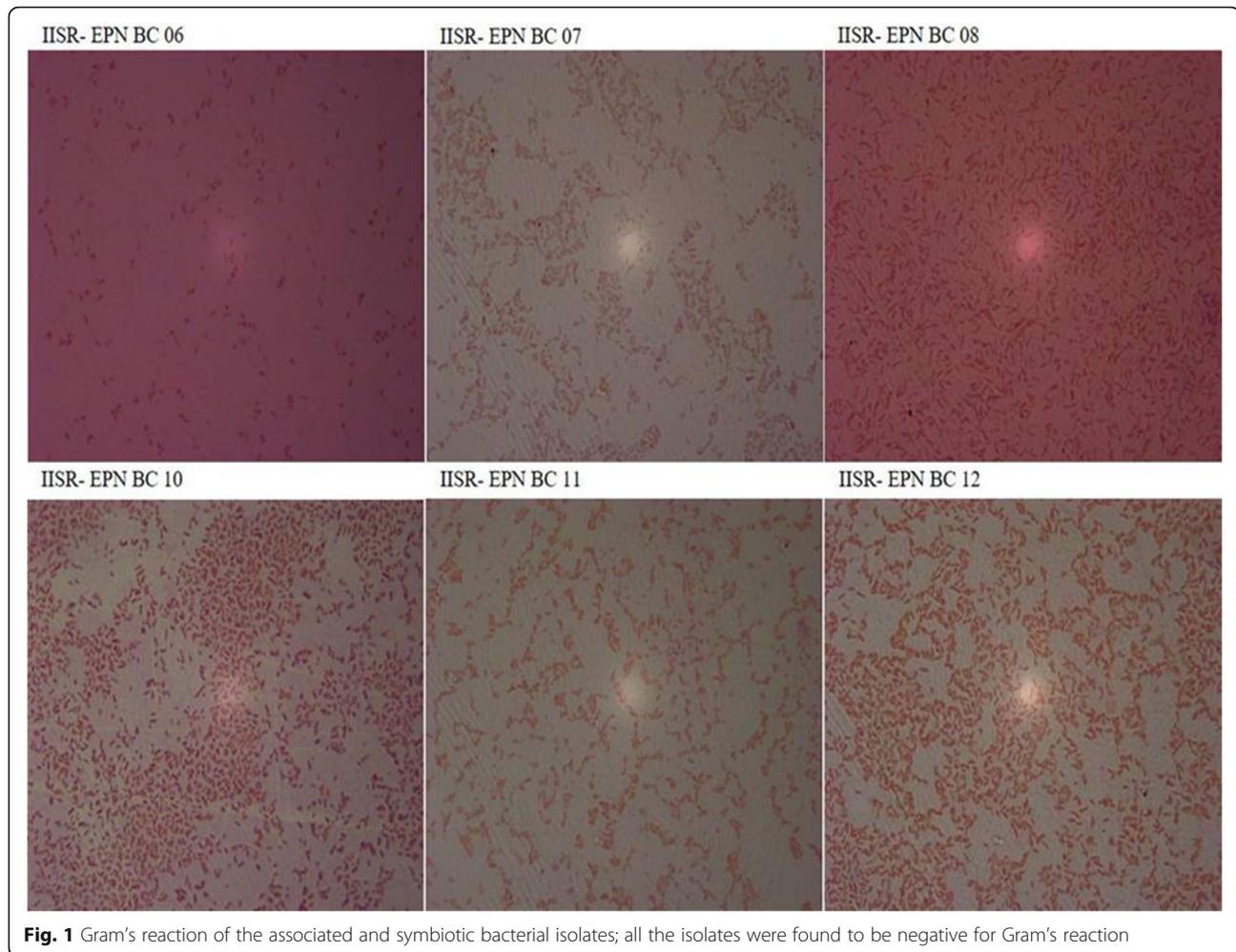


Fig. 1 Gram's reaction of the associated and symbiotic bacterial isolates; all the isolates were found to be negative for Gram's reaction

analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1204 positions in the final dataset (Fig. 3 b).

Alcaligenes faecalis (IISR-EPN BC 08 and 11)

A. faecalis is a gram-negative, rod-shaped, motile, non-nitrate reducing, oxidase positive, catalase positive, alpha hemolytic, and citrate-positive obligate aerobe that is commonly found in the environment. *A. faecalis* belong to phylum Proteobacteria, class Betaproteobacteria, exploration of symbiotic and associated bacteria order Burkholderiales, and family Alcaligenaceae. IISR-EPN BC 08 and 11 shows 99% similarity *A. faecalis* subsp. *parafaecalis* strain ALK518 (accession no. KC456571) and *A. faecalis* subsp. *parafaecalis* strain ALK517 (accession no. KC456570). The optimal tree with the sum of branch length was 11.34437646. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

The analysis involved 7 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1155 positions in the final dataset (Fig. 3 c).

Pseudomonas entomophila (IISR-EPN-BC 10)

Pseudomonas entomophila is a strain of bacterium that lives in the soil, aquatic, and rhizosphere environment soil and can infect insects. It is a gram-negative rod, facultative anaerobe, positive for catalase, oxidase, Voges-Proskauer test, and citrate utilization, whereas, it is negative for indole production, methyl red, and urease. IISR-EPNBC 10 shows 100% similarity with *P. entomophila* strain 2P25 (accession no. EF178450), *P. entomophila* strain L48 (accession no. CT573326), and *P. entomophila* strain L48 (accession no. NR115336). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were

Table 3 Biochemical characteristics of associated and symbiotic bacterial isolates

	Bacteria isolates					
	IISR-EPN BC 06	IISR-EPN BC 07	IISR-EPN BC 08	IISR-EPN BC 10	IISR-EPN BC 11	IISR-EPN BC 12
Biochemical tests						
Catalase test	+	+	+	+	+	+
Oxidase test	-	-	-	+	-	-
Indole test	-	-	-	-	-	+
Methyl red test	-	-	-	-	-	+
Voges-Proskauer test	-	+	+	+	+	-
Citrate utilization test	+	+	+	+	+	+
Urease test	+	-	-	-	-	-
H ₂ S production	-	+	-	-	-	-
Proteolysis test	+	-	-	+	-	-
Esculin hydrolysis	-	-	-	-	-	-
Lipolysis test	+	-	-	+	-	-
Carbohydrate fermentation						
Glucose	+	+	+	+	+	+
Lactose	-	-	-	-	-	-
Sucrose	-	-	-	-	-	+
Maltose	-	+	+	+	+	+
Mannitol	-	+	+	-	+	+

+ positive, - negative

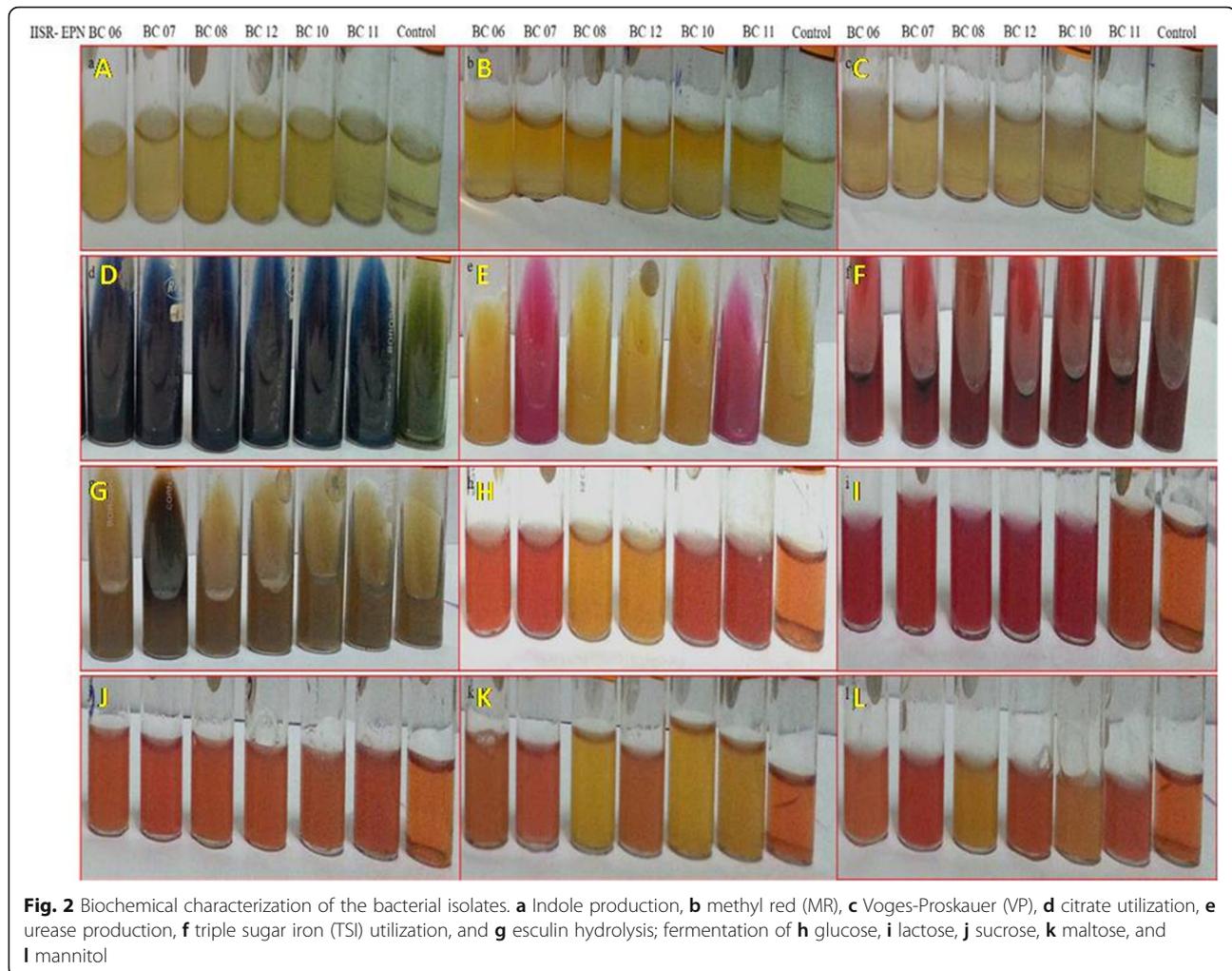
eliminated. There were a total of 747 positions in the final dataset (Fig. 3 e).

***Photorhabdus luminescens* (IISR-EPN BC 12)**

Photorhabdus is a genus of bioluminescent, gram-negative bacilli which lives symbiotically within entomopathogenic nematodes, hence the name *photo* (which means light-producing) and *rhabdus* (rod shaped) with some bipolar staining and vacuolation with occasional long filamentous forms. *P. luminescens* strain IISR-EPN BC 12 was facultative anaerobe, positive for catalase, indole production, citrate utilization, and methyl red, whereas it was negative for oxidase, urease, and Voges-Proskauer test. IISR-EPN BC 12 shows 100% similarity to *P. luminescens* subsp. *akhurstii* strain 0805-P5G (accession no. EU301784), *P. luminescens* subsp. *akhurstii* strain FRG04 (accession no. NR028869), and *P. luminescens* subsp. *akhurstii* strain 0813-124 (accession no. DQ223040). The optimal tree with the sum of branch length was 20.34437646. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 957 positions in the final dataset (Fig. 3 d).

This indigenous EPN *Heterorhabditis* sp. (IISR-EPN 09) was found to be effective against various economically important insects pests like the shoot borer *Conogethes punctiferalis*, hairy caterpillar *Euproctis* sp., cardamom root grub *Basilepta fulvicorne*, semi-looper *Synegia* sp., and leaf feeder *Lema* sp. (Pervez et al. 2014b, 2016, and Pervez et al. 2017).

The present study led to the identification of 5 bacterial species viz., *P. luminescens*, *P. vermicola*, *A. faecalis*, *A. aquatilis*, and *P. entomophila* on the basis of phenotypic, biochemical, and molecular characterizations. The nucleotide sequences of 16S rRNA genes of these isolates were submitted to GenBank and assigned accession numbers MH249776 to MH249780. Among them, *P. luminescens* is a well-known symbiotic bacterium and the rest are associated bacteria. Previously, associated bacteria from genera other than the well documented *Photorhabdus* and *Xenorhabdus* have been reported to be present in or on the surface of EPNs. Torres-Barragan et al. (2011) reported the presence of 4 bacterial species viz. *Serratia marcescens*, *Enterococcus mundtii*, *Achromobacter xylosoxidans*, *Providencia rettgeri* from the EPN, and *Oscheius carolinensis*. *P. entomophila* has been found to be toxic against the larvae and adults of *Drosophila melanogaster* and some insects belonging to 3

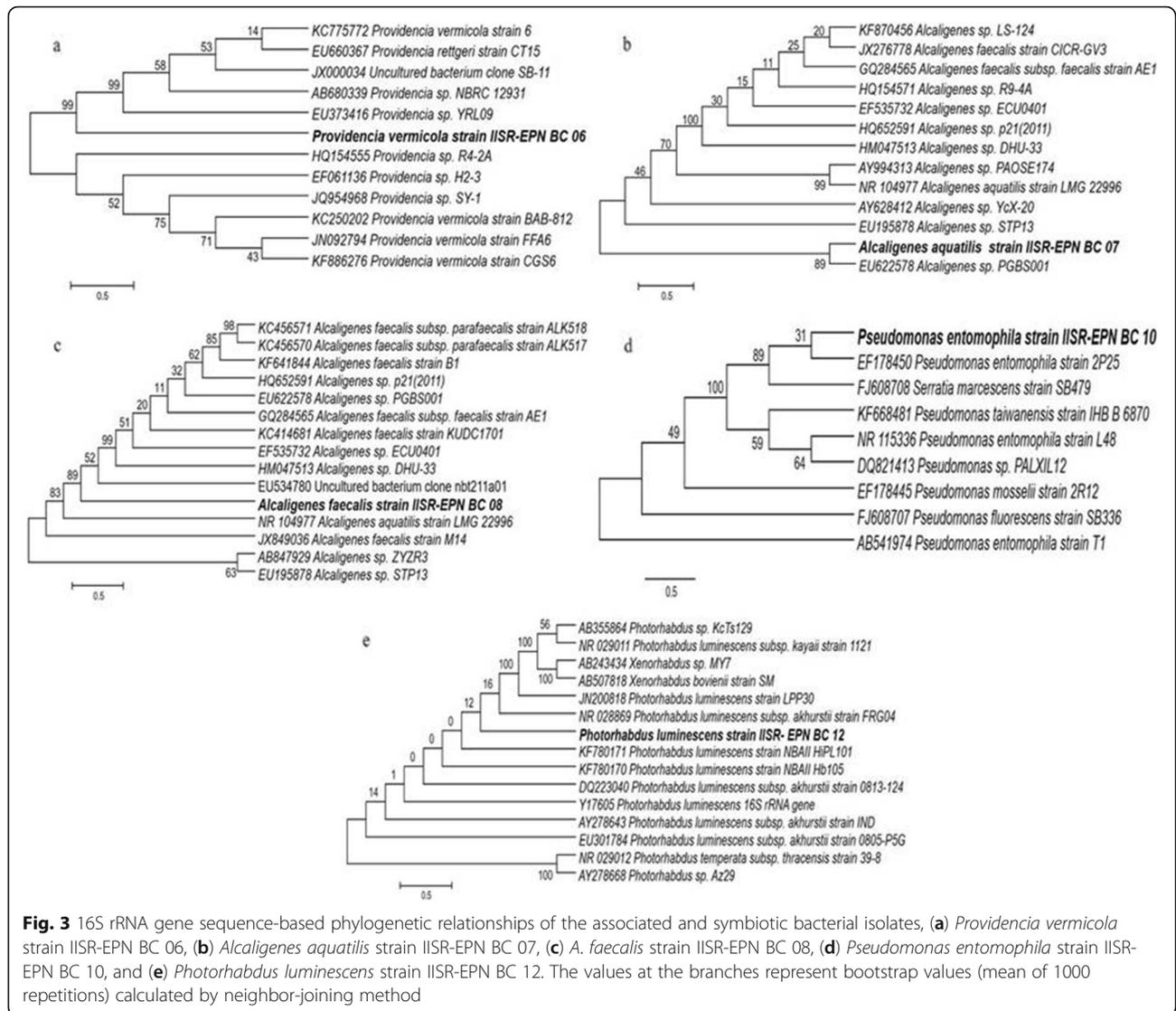


different orders (Vodovar et al. 2006; Ruiu 2015). For a long time, the discovery of EPN-associated bacteria more popularly referred to as “frequently associated microbiota (FAM)” has often been dismissed as laboratory/handling contaminants; but as they became frequently encountered (Walsh and Webster 2003; Gouge and Snyder 2006, and Singh et al. 2014) in the hemolymph of infected insects, researchers started paying attention to them. FAM consists predominantly of members of Proteobacteria, while other genera are also present, except for a few of them like *P. entomophila*, their role in killing the insect host has not been established (Ogier et al. 2020). However, their co-existence with the core symbionts even in nematodes that have been maintained for generations in laboratory conditions indicates that they may have some important roles to play, which may be discovered in future. In fact, it is now widely debated whether the pathogenicity of IJs is exclusively because of the core symbionts

(*Xenorhabdus* and *Photorhabdus*) or it is a combined effect of core symbiont and FAM (Ogier et al. 2020); such studies point towards the involvement of a rather “pathobiome” and the involvement of more than one pathogen in establishing a disease as against the widely believed concept of one pathogen one disease.

Conclusion

In the present study, six bacterial isolates from the *Heterorhabditis* sp. (IISR-EPN 09) were identified and the latter is native to the spice plantation of the southern India. The bacteria were identified based on the phenotypic-biochemical criteria and the sequencing of 16S rRNA gene. Since the relationship between the symbiotic bacterium and the nematode is highly specific, *Photorhabdus* sp. from the *Heterorhabditis* sp., additionally some associated bacterial species belonging to genera other than *Photorhabdus*, were isolated and identified. These species were not reported to have a symbiotic relationship with



Heterorhabditis sp. However, some associated bacteria have been found to augment the toxicity of the EPNs in some previous studies. Evaluation of the entomopathogenic potential of the symbiotic and associated bacteria against the insect pests shall be carried out to explore their toxicity potential.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s41938-020-00343-9>.

Additional file 1: Supplementary Fig. 1.

Abbreviations

ICAR: Indian Council of Agricultural Research; IISR: Indian Institute of Spices Research; EPNs: Entomopathogenic nematodes; IJs: Infective juveniles; ITS: Internal transcribed spacer; rRNA: Ribosomal ribonucleic acid; PCR: Polymerase chain reaction; BOD: Biological oxygen demand

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Authors' contributions

RP: research concept and design, collection and/or assembly of data, data analysis and interpretation, writing the article, critical revision of the article. SAL: research concept and design, data analysis and interpretation. SP: data analysis and critical revision of the article. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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