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Article

16S rDNA-Based Phylogeny of Non-Symbiotic Bacteria of Entomopathogenic Nematodes from Infected Insect Cadavers

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Abstract

Using 16S rDNA gene sequencing technique, three different species of non-symbiotic bacteria of entomopathogenic nematodes (EPNs) (*Steinernema* sp. and *Heterorhabditis* sp.) were isolated and identified from infected insect cadavers (*Galleria mellonella* larvae) after 48-hour post infections. Sequence similarity analysis revealed that the strains SRK3, SRK4 and SRK5 belong to *Ochrobactrum cytisi*, *Schineria larvae* and *Ochrobactrum anthropi,* respectively. The isolates *O. anthropi* and *S. larvae* were found to be associated with *Heterorhabditis indica* strains BDU-17 and Yer-136, respectively, whereas *O. cytisi* was associated with *Steinernema siamkayai* strain BDU-87. Phenotypically, temporal EPN bacteria were fairly related to symbiotic EPN bacteria (*Photorhabdus* and *Xenorhabdus* genera). The strains SRK3 and SRK5 were phylogeographically similar to several non-symbionts and contaminated EPN bacteria isolated in Germany (LMG3311T) and China (X-14), while the strain SRK4 was identical to the isolates of *S. larvae* (L1/57, L1/58, L1/68 and L2/11) from *Wohlfahrtia magnifica* in Hungary. The result was further confirmed by RNA secondary structure and minimum energy calculations of aligned sequences. This study suggested that the non-symbionts of these nematodes are phylogeographically diverged in some extent due to phase variation. Therefore, these strains are not host-dependent, but environment-specific isolates.

Key words: entomopathogen, *Schineria*, *Ochrobactrum*, non-symbionts, phylogenetics, phase variation

Introduction

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* are associated with mutualistic bacterial symbionts, the gamma Proteobacterium of *Xenorhabdus* and *Photorhabdus*. Since symbiotic bacteria are toxic to insects, the insect immune system is competent to eliminate them proliferating within the insect body (*1*-*3*). Symbiotic bacteria multiply rapidly inside the host and produce some structural and antibacterial compounds (xenocoumarins, xenorhabdins, bacteriocins, *etc.*), which overcome the host immune system, contaminate bacterial growth and their competitors (*4*, *5*).

Several non-symbiotic bacteria have been identified from the nematode-infected insect cadavers, some of which have been studied in detail for their survival and physiological variation. For example, *Alcaligenes* sp., *Pseudomonas aureofaciens*, *Pseudomonas fluorescens*, *Enterobacter agglomerans*, *Serratia liquefaciens* and *Acinetobacter* sp. are temporal associated bacteria isolated from nematode *Stein-*

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ernema carpocapsae (*6*, *7*). Similarly, *Ochrobactrum anthropi*, *Paracoccus denitrificans* and *Pseudomonas maltophilia* have been found to be associated in *Steinernema scapterisci* (*8*, *9*). *Acinetobacter* sp., *Paenibacillus nematophilus* and some other microbes in the insect cadavers also adhere to the surface of the nematodes (*10*, *11*). Certain bacterial symbionts exist in the insect host to increase the pathogenic potential of EPNs and their associating bacteria; however, it has not been studied elaborately.

Among temporal associated EPN bacteria, *O. anthropi* is a ubiquitous organism, which is widely distributed in the environment and water sources (*12*) as well as in the hemolymph of insect *Galleria mellonella* infected by *Heterorhabditis* nematodes (*13*). *Ochrobactrum* strain acts as opportunistic pathogens in human (*14*). Biochemical and molecular (16S rDNA sequencing) taxonomy studies revealed that *O. anthropi* and *O. intermedium* are resembled to symbiotic bacteria *Photorhabdus luminescens* subsp. *akhurstii* isolated from *Heterorhabditis indica* (*15*, *16*). *Schineria larvae*, a member of the gamma Proteobacteria, is associated with *Wohlfahrtia magnifica* (Diptera: Sarcophagidae), an obligate fly larval parasite (*17*).

A wide range of non-symbionts have been associated with EPN bacteria and EPNs infecting different groups of insects. However, their physiological roles, host specificities, surveillances and phylogenetic relationship are still unknown. In the present investigation, we aimed to isolate and identify the EPN non-symbiotic bacteria from *G. mellonella* larvae after 48-hour post infections. Furthermore, we analyzed the phylogenetic relatedness of these isolates with other non-symbiotic bacteria from various countries based on their 16S rDNA gene sequences.

Results

Three temporal bacterial strains were isolated from the cadaver of insect *G. mellonella* infected by EPNs *Steinernema siamkayai* and *H. indica*. Morphological characteristics of these strains, including size, shape and motility, were similar to those of the bacterial members in Enterobacteriaceae family. According to their biochemical characteristics, the bacterial isolates were grouped into the genera of *Ochrobactrium* and *Schinaria*. Some phenotypic features of them were resembled to the characters of EPN-symbiotic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. (**Table 1**). 16S rDNA gene fragments amplified from total DNA of these organisms were approximately 1,500 bp in length. Sequence similarity analysis implied that the sequences of isolates SRK3 and SRK5 were closely

Table 1 Morphological and biochemical characteristics of bacterial isolates of *Steinernema* and *Heterorhabditis* spp. infected *G. mellonella* and the most closely phylogenetically related species of the genera *Xenorhabdus* and *Photorhabdus*

Characters	*O. anthropi*	*O. cytisi*	*S. larvae*	*Xenorhabdus*	*Photorhabdus*
Gram stain	−ve	−ve	−ve	−ve	−ve
Cell shape	Rod	Rod	Rod	Rod	Rod
Motility	Yes	Yes	Yes	Yes	Yes
Citrate	−	−	−	−	−
H_2S production	−	+	−	+	−
VP	−	−	−	−	−
Indole	−	−	−	−	−
Urease	+	+	+	−	−
Oxidase	+	+	+	−	−
Catalase	+	+	+	−	+
Gelatin	−	−	−	−	+
Nitrate reduction	+	+	+	+	−
Glucose	+	+	+	+	+
Sucrose	+	+	+	−	−

Note: "+" denotes positive; "−" denotes negative.

related (100% identity and 2,666 alignment score) to organisms belonging to *O. cytisi* (AM11072) and *O. anthropi* (AY513494), respectively, whereas isolate SRK4 belonged to *S. larvae*.

As shown in **Figure 1A**, *Ochrobactrum* genus formed two major clusters in the phylogenetic tree, where isolates SRK3 and SRK5 were in a separate monophyletic cluster containing different strains of *O. cytisi* and *O. anthropi* with bootstrapping value 963. The different strains of *O. tritici* also formed a separate monophyletic cluster, showing that it is distantly related with other species. *Brucella* sp. DB-6 was used as an outgroup organism to generate an optimal phylogenetic classification of the isolates. Therefore, isolates SRK3 and SRK5 were confirmed as *O. cytisi* and *O. anthropi*, respectively. In **Figure 1B**, *Ferritrophicum radicicola* strain CCJ, *Dokdonella* sp. PYM5-8, *Aquimonas* sp. D11-34A and UK-29,

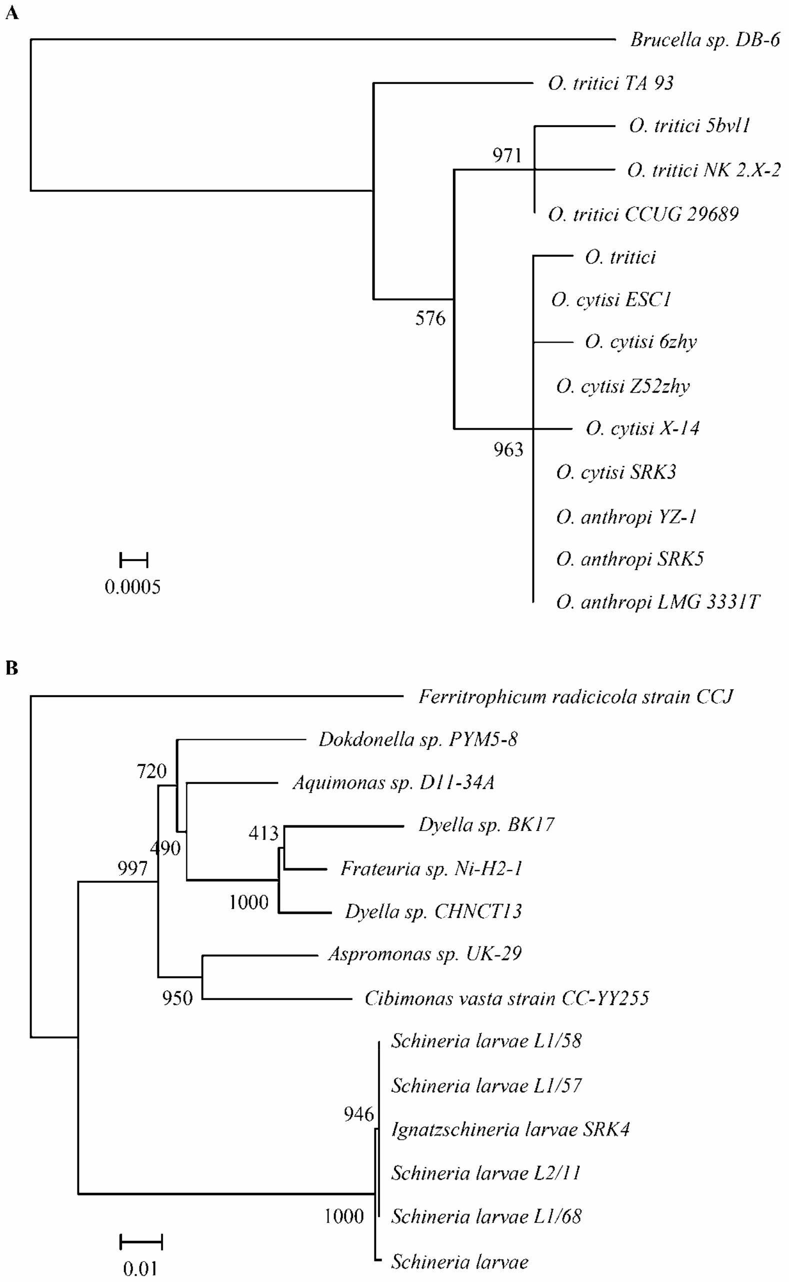

Figure 1 Phylogenetic tree of *O. anthropi*, *O. cytisi* (**A**), *S. larvae* and closely related species (**B**) based on 16S rDNA gene sequences.

Dyella sp. BK17 and CHNCT13, *Frateuria* sp. Ni-H2-1 and *Cibimonas vasta* strain CC-YY255 were used as the outgroups for the phylogenetic classification of isolate SRK4. Among them, *F. radicicola* strain CCJ was chosen as a suitable outgroup organism to reveal the ancestral relationship of isolate SRK4. In the phylogenetic tree, all of the outgroup organisms except *F. radicicola* strain CCJ formed one cluster, whereas *S. larvae* and isolate SRK4 were grouped into the other (bootstrapping value 946) and formed a monophyletic clade. Consequently, isolate SRK4 was phylogenetically close to the members of the genus *S. larvae*.

The isolates SRK3 and SRK5 phylogeographically corresponded to the similar isolates from Spain, China, Germany, France and Portugal. However, many of them have shown closer relationships to *O. anthropi* and *O. tritici* isolated from soil and sludge in China and Germany (**Table 2**). Interestingly, the isolate SRK4 in this study was geographically resembled to the *S. larvae* strains (L1/57, L1/58, L1/68 and L2/11) isolated from *W. magnifica* in Hungary. As a significance of close phylogenetic proximity, RNA secondary structure and minimum energy calculations of these sequences were performed to reveal the evolution of corresponding structural constraints and energy conformers (**Figures 2** and **3**). The results showed that 16S rDNA sequences of isolates SRK3 and SRK5 generated energetically favorable RNA secondary structures. Several loop structures and loop energies were differed in these strains compared with those of phylogenetically related organisms. The free energy of structure in SRK3 and SRK5 was −341.3 kcal/mol and −346.6 kcal/mol, respectively, both were closely related to the free energy of structure in *O. anthropi* strain LMG3331T (−333.6 kcal/mol) from Germany. The free energy of structure in strain SRK4 (−286.5 kcal/mol) were related to that of four strains of *S. larvae* (L1/57, L1/58, L1/68 and L2/11) from Hungary, in which the free energy of structure in strain L2/11 (−297.6 kcal/mol) showed a significant relatedness to strain SRK4.

Table 2 Phylogeographic distribution and isolation sources of non-symbiotic bacteria of EPNs

Accession	Strain	Source	Geographical location
O. cytisi			
EU826069	SRK3	Insect hemolymph	India
O. anthropi			
EU826071	SRK5	Insect hemolymph	India
AY776289	ESC1	*Cytisus scoparius*	Spain
AM411072	6zhy	Deep sea bacterium	China
EU187495	X-14	Quinoline-degrading biofilm	China
AM114398	LMG 3331T	–	Germany
AJ867290	SAIII104	Wheat rhizoplane	France
AJ867295	LMG 3331	–	Germany
O. tritici			
EU301689	Y13	Soil	China
EU870448	PBQ-H2	Pesticide plant sludge	China
EU352762	NK 2.X-2	–	China
AY429607	5bvl1	Activated sludge	Portugal
AM114403	CCUG 29689	–	Germany
AM490635	TA 93	–	Germany
S. larvae			
EU826070	SRK4	Insect hemolymph	India
EF120377	Romans	–	France
AJ252143	L1/68	*W. magnifica*	Hungary
AJ252144	L1/57	*W. magnifica*	Hungary
AJ252145	L1/58	*W. magnifica*	Hungary
AJ252146	L2/11	*W. magnifica*	Hungary

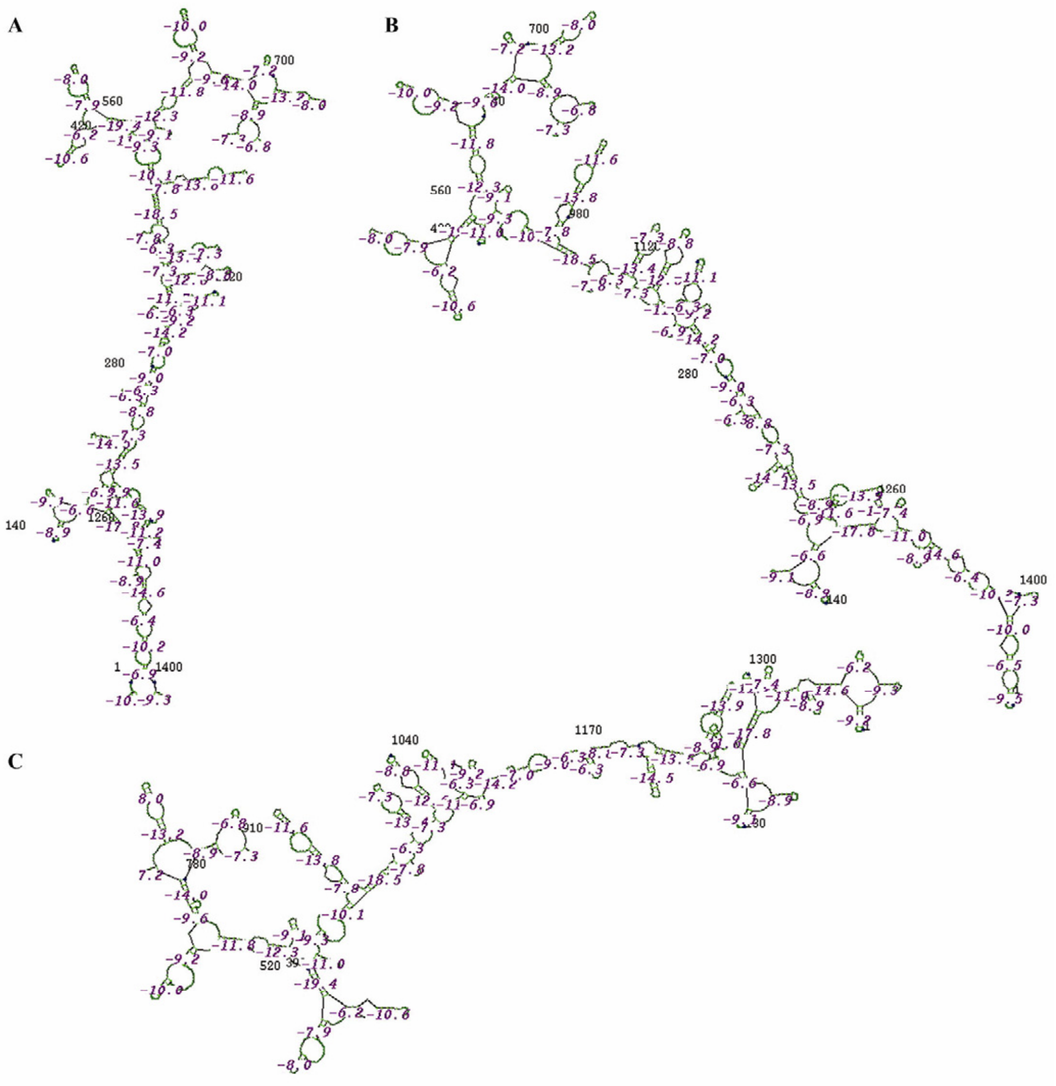

Figure 2 Graphical depiction of the predicted minimum free energy secondary structure for the sequences of strains SRK3 (**A**), SRK5 (**B**) and of reference strain LMG3331T (**C**).

Discussion

Bacterial cells alone are generally unable to access insect hemolymph, unless they are inoculated or injected into the insect body. Bacterial symbionts multiply rapidly within the host and produce a variety of anti-microbial compounds to suppress the growth of contaminants or competing pathogens (*4*, *5*, *18*). Herein, we have isolated three different species of bacterial colonies from hemolymph of *G. mellonella* and identified them as *O. anthropi*, *O. cytisi* and *S. larvae*. Phase variation is common in bacterial species; phase I variants offer ideal nutrient supply to the associated nematode and produce a variety of antibiotic compounds (*7*). Ideally, the temporal bacteria may support the growth of EPN bacterial endosymbionts by supplying nutrients (to access on degrading macromolecules) from infected insect cadavers. Hence, such bacteria can escape from antibacterial compounds produced by symbiotic bacteria and survive in insect host.

Bacterial non-symbionts can be isolated from insects after nematode exposure of less than 6 h or more than 42 h (*7*). In this study, three non-symbiotic bacteria were isolated from hemolymph of insect *G. mellonella* infected by indigenous species of EPNs *H. indica* and *S. siamkayai* in the Western Ghats of South India. However, the growth of these organisms could be suppressed subsequently by the action of antibiotic compounds produced from bacterial symbionts (phase

Figure 3 Graphical depiction of the predicted minimum free energy secondary structure for the sequences of strain SRK4 (**A**) and of reference strains L1/57 (**B**), L1/58 (**C**) and L2/11 (**D**).

I variant) (*7*). Even so, Babic *et al* (*13*) reported the occurrence of natural dixenic association between the bacterial symbiont *P. luminescens* and the bacteria related to *Ochrobactrum* sp. in 33% of *Heterorhabditis* species. In this study, we also found the natural dixenic association of symbiont *P. luminescens* subsp. laumondii T101 with non-symbionts *O. anthropi*, *O. cytisi* and *S. larvae* in EPNs.

Phenotypic characteristics of collected species can be useful for species identification. We have shown that the non-symbionts were phenotypically related to symbionts of EPNs *H. indica* and *S. siamkayai*, as agreed to Babic *et al* (*13*). A variety of Gram-negative bacteria have been shown to support reproduction of steinernematid nematodes and are pathogenic to insect host, but might play a role in the nutritional uptake of nematodes from degrading tissues of insect cadaver (*2*, *3*, *19*). *Ochrobactrum* sp. was isolated in the gut region of termites and was reported to be involved in the degradation of hemicelluloses (*20*). Therefore, the occurrence of these secondary bacterial associates could cohabitate in the insect during parasitism, and might enhance the host mortality induced by primary symbionts. When axenic nematodes are introduced into an insect host in the presence of a variety of non-symbiotic bacteria, nematodes are able to reproduce in the absence of the symbionts (*2*, *3*). Thus, the occurrence of non-symbionts in association with nematodes in the insect host can be sustained for nematode reproduction and nutritional uptake even in the absence of symbionts. Such physiological events occur due to ancestral adaptations to microbivorous behavior (*3*).

Ochrobactrum sp. was phylogenetically related to the members in the genus of *Brucella*, belonging to the alpha-2 subdivisions of Proteobacteria (*21*). *F. radicicola* strain CCJ was already reported as an outgroup organism in the taxonomic classification of *S. larvae* (*22*). Similarly, both outgroup organisms were found to serve as ancestors on enlightening the evolutionary relatedness of strains SRK3, SRK4 and SRK5. Recent taxonomic studies have resulted in the description of an increasing number of new taxa involved in identification of same genera in insect pathogens. Thus, phylogenetic analysis of this study determines some characteristics useful for species identification from insect–nematode–bacterial complex.

According to Poinar's hypothesis, the bacterial contaminants have been originated from insect guts by indiscriminated feeding of nematodes within the host (*23*). Herein, the strains SRK3 and SRK5 are closely related to free energy of structure in *O. anthropi* strain LMG3331T (−333.6 kcal/mol) from Germany whereas the strain SRK4 is related to *S. larvae* L2/11 (−297.6 kcal/mol) from Hungary. Similar observations were reported for *S. scapterisci* Nguyen and Smart, which was transferred from South America and subcultured many times in Florida. This nematode was associated with *O. anthropi*, *P. denitrificans*, *P. maltophilia*, and *Xenorhabdus* sp. (*7*-*9*). Consequently, non-symbiotic bacteria could be transferred from Germany and Hungary toward host nematodes or insects isolated in the agro-ecosystem of Western Ghats of South India. Overall, we proposed that specific associating bacterial species require firm observation for the mass production of EPNs in biological control of insect pests.

Materials and Methods

Insect and nematode culture

The greater wax moth larvae *G. mellonella* (Pyralidae, Lepidoptera) was used for nematode baiting and the multiplication of nematodes *S. siamkayai* (Bdu-87) and *H. indica* (Bdu-17 and Yer-136) isolated from Western Ghats of South India. Initially, eggs were obtained from the Department of Biotechnology, Bharathidasan University, India, and were kept in rearing plastic boxes with artificial diet. Insects were maintained in aerated plastic containers (32.5×17.6×10 cm) at (25±2)°C. The nematode was cultured in the late instar larvae of *G. mellonella* according to the method described by Woodring and Kaya (*24*). An infective juvenile was stored at a concentration of approximately 1,000-4,000 per mL in distilled water with 0.1% formalin in the tissue culture flask at 19-20°C in BOD incubator.

Isolation of bacterial non-symbionts from insect hemolymph

Non-symbiotic bacteria of each nematode species were isolated from infected larvae of *G. mellonella*. Late instar *G. mellonella* were placed on the surface of a filter paper in 35-mm petri dishes. Individual nematodes were transferred onto a filter paper surface at a dose rate of 400 per petri dish. All the dishes were sealed with para film, and then incubated at 25ºC for 24 h. Thereafter, the larvae were removed, rinsed in distilled water and surface sterilized with 70% ethanol, and left to drying in a laminar flow cabinet. Hemolymph was obtained by dissecting dorsally between the 5th and 6th interstitial segments, and was collected with a sterile loop and streaked on NBTA agar (nutrient agar supplemented with 25 mg bromo thymol blue and 40 mg triphenyltetrazolium chloride per liter) plates (*24*) and then incubated at 28°C for 48 h. Cell morphology and motility of the isolated bacterial colonies were studied by direct contact and phase-contrast microscopy. Gram staining and biochemical characteristics of these isolates were carried out according to the methods described in Bergey's manual (*25*).

DNA extraction, PCR amplification and sequencing

The bacteria were cultured in Tryptic Soy Broth (TSB) at 25°C for 48 h. The bacterial cells were washed three times with sterilized distilled water by centrifuging at 4,000 rpm for 2 min at 4°C. Total DNA was extracted using a DNA isolation kit (Genei, India). 16S rDNA gene amplification was done by a PCR gradient thermocycler (Eppendorf, India) using forward primer 5′-AGAGTTTGATCCTGGCTCAG and reverse primer 3′-GACGGGCGGTGTGTACAA. The total volume of a PCR mixture was 50 μL, containing 5 μL of 10× PCR buffer, 8 μL dNTP mixture, 2.5 μL Taq DNA polymerase, 2 μM of each primer, and 100 ng of template DNA. The PCR reaction mixture condition was 94ºC for 2 min for initial denaturation, followed by 35 cycles of 94ºC for 1 min, 52ºC for 1 min, 72ºC for 2 min and final extension at 72ºC for 7 min. PCR products were separated in a 2% agarose gel (containing 0.5 mg/mL ethidium bromide) electrophoresis and then visualized under gel documentation. PCR products were purified using PCR purification kit (Genei, India). The 16S rDNA gene sequencing was performed by nucleic acid automatic ordering meter (ABI 3130 Genetic Analyzer).

Phylogenetic analysis

To obtain similarity sequences for sequenced PCR products, NCBI BLASTn search tool (*26*) was used to retrieve sequences from GenBank of NCBI and RDP-II (Ribosomal database project) (*27*). 16S rDNA sequences of bacteria were aligned using the Clustal X program (*28*). This alignment was the basis for the phylogenetic analysis of the sequence data with different methods. Every aligned sequence was inspected manually and unreliable sequences were deleted. Phylogenetic calculations were made according to a neighbor joining algorithm implemented in MEGA 4.0 software (*29*) by applying an "evolutionary model", which infers different evolutionary rates at different sites. Bootstrap analyses with 1,000 resamplings were performed to obtain estimates of phylogenetic tree topologies for all methods. Concerning the importance of the DNA sequence alignment, RNA secondary structure and minimum energy calculations were performed by GeneBee-NET program (*30*, *31*) using a greedy algorithm. The parameters were set as: energy threshold = −4.0; cluster factor =2; conserved factor =2; compensated factor =4; and conservativity =0.8. The resulting alignment was treated in the same way as the Clustal X alignment. The calculated trees based on GeneBee-NET alignment showed branching patterns highly similar to the Clustal X alignment-based trees. Therefore, the results presented for trees were calculated after alignment with Clustal X only.

Nucleotide sequence accession numbers

The partial 16S rDNA gene sequences determined in this study have been deposited in the GenBank of NCBI database under accession numbers EU826069 to EU826071.

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Authors' contributions

MR, RKR and KP carried out the experimental study. MR and PC prepared the manuscript and helped data collection and phylogenetic analysis. SS supervised the research and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

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