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Plant Protection

 ISSN: 2617-1287 (Online), 2617-1279 (Print)
<http://esciencepress.net/journals/PP>

MOLECULAR IDENTIFICATION AND GROWTH OF *XENORHABDUS* AND *PHOTORHABDUS* SYMBIONTS OF ENTOMOPATHOGENIC NEMATODES

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ARTICLE INFO

Article history

Received: 3rd July, 2022Revised: 6th July, 2022Accepted: 9th August, 2022

Keywords

*Xenorhabdus bovienii**Photorhabdus**luminescens*

Growth

molecular identification

Phylogeny

ABSTRACT

From the characterisation, the partial 16 S gene sequences obtained for the two bacteria were subjected to blast-bootstrap analysis to obtain the phylogenetic tree. In assessing the similarity of *Xenorhabdus bovienii* with five other *Xenorhabdus* spp., it was found to be 96% similar to *X. nematophila* and *X. japonica* with Accession Numbers D78006 and NR027194 respectively. The *X. bovienii* was closer to *X. beddingii* 95% and *X. kazodoii* and 97% similarity to *X. poinarii*. From the phylogenetic tree, the two species of bacteria were found to belong to the genera *Xenorhabdus* and *Photorhabdus*. The two bacteria were compared with *Xenorhabdus japonica* NR027194, *X. nematophila*, D78006 *X. poinarii* DQ211703, *X. beddingii* AY278675 and *X. kozodoii* Eu 190977. The results showed that at 20 °C both the bacteria *X. bovienii* and *Photorhabdus luminescens* increased to a log concentration of 7.5 after 6 h. They then increased slightly up to 15 h. At 25 °C *X. bovienii* had a concentration of 6.1 after 1 h but only increased slowly to 6.6 after 15 h. However, *P. luminescens* started with 5.6 after 1 h but increased smoothly to 7.5 after 15 h. *X. bovienii* and *P. luminescens* at 30 °C had a concentration of 6.2 after 1 h.

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INTRODUCTION

Bacteria are classified and identified to distinguish among strains and to group them by criteria of interest to microbiologists and other scientists. Bacteria are named so that investigators can define and discuss them without the necessity of listing their characteristics. The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. Several bacteria share the same or similar phenotypic characteristics. Parameters such as colour, growth rate,

shape, presence or absence of flagella, smell/odour, media and temperature for growth/propagation, dye adsorption and antimicrobial production might be similar to many bacteria belonging to different genera or species. Andreoglou (2001) observed that both *Pseudomonas oryzihabitans* and *Xenorhabdus nematophila* produce deep blue coloration on nutrient bromothymol blue-triphenyl tetrazolium chloride agar (NBTA agar). Boemare and Akhurst (1988) observed that several species of *Xenorhabdus* show optimum growth around 25 °C. Boemare (2002) reported that *X.*

nematophila and *X. japonica* have 35 °C as the upper threshold for growth and cells of all species of *Xenorhabdus* are asporogenous, rod-shaped and gram negative. Currently, comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique as the 16S rRNA gene sequence analysis can better identify poorly described rarely isolated or phenotypically aberrant strains and can be routinely used for the identification of mycobacteria, and can lead to the recognition of novel pathogens and non-cultured bacteria (Clarridge, 2004). Problems remain in that the sequences in some databases are not accurate, there is no consensus to the quantitative definition of genus or species based on 16S rRNA gene sequence data, the proliferation of species names based on minimal genetic and phenotypic differences raises communication difficulties, and micro heterogeneity in the 16S rRNA gene sequence within a species is common. Despite its accuracy, 16S rRNA gene sequence analysis lacks widespread use beyond the large and reference laboratories because of technical and cost considerations. Thus, a future challenge is to translate information from 16S rRNA gene sequencing into convenient biochemical testing schemes, making the accuracy of the genotypic identification available to the smaller and routine microbiology laboratories (Tailliez et al., 2006).

The development and use of entomopathogenic nematodes, is one of the important strategies among different biological control agents to manage insect pests ecofriendly (Gulzar et al., 2020; Rahoo et al., 2016a; Rahoo et al., 2019a; Rahoo et al., 2019b; Rahoo et al., 2016b). Among all the nematodes tested for the management of insect pests, the members of the families Heterorhabditidae and Steinernematidae have been widely investigated for their efficacy against a number of insect pests as they have many advantages over synthetic detrimental chemicals (Rahoo et al., 2018b; Rahoo et al., 2017b; Rahoo et al., 2017a; Rahoo et al., 2018a). The efficacy of these nematodes is attributed to the bacteria in their guts which cause septicemia and kill insect pests (Rahoo et al., 2011). In addition to these bacteria, many entomopathogenic fungi are also responsible for controlling various insect pests (Javed et al., 2019a, 2019b; Shehzad et al., 2022; Shehzad et al., 2021).

Xenorhabdus spp. and *Photorhabdus* spp. have shown efficacy towards insect pests especially the soil-dwellers.

Each bacterium has temperature regimes at which growth is optimised. Boemare (2002) summarized the temperature requirements of *Xenorhabdus* spp. and *Photorhabdus* spp. The upper threshold for growth of *X. bovienii* was reported to be 32 °C and for *P. luminescens* 35-39°C. An understanding of the optimum temperature regimes at which the bacteria grow is essential. If the temperature of growth lies within the temperature range of soil in the tropics, then applied bacteria would be able to survive and control the soil-dwelling insect pest. Secondly, Divakar and Pawar (1987) reported that soil temperatures between 25 and 35 °C were essential for the life cycle development of major cutworms in Asia in summer season and 15 to 20 °C in winter season. The cut worm is adapted to these temperature regimes so the bacteria to be used in the control must survive at the same or similar temperature. Since soil temperatures up to 30°C are common in the tropical soils of Sindh province in Pakistan cut worms thrive at temperatures between 15 and 35 °C.

In this study, entomopathogenic bacteria were isolated from infective juveniles of *Steinernema feltiae* and *Heterorhabditis bacteriophora* which were already maintained in the laboratory at the Department of Agriculture. Hitherto no attempt had been made to confirm the identity of the bacteria in the population of *S. feltiae* originating from the University campus. Whilst it is likely that *X. bovienii* and *P. luminescens* were the dominant symbionts in *S. feltiae* and *H. bacteriophora* respectively, work by Elawad (1998) had shown that other genera of bacteria can be associated with entomopathogenic nematodes.

Therefore, the objectives of this study were to confirm whether the standard bacteria used in this study was *Xenorhabdus boveinii* and *Photorhabdus luminescens* to determine and confirm gram-negative bacteria obtained from available suspension to establish any similarity among the bacteria and to determine the growth of *X. boveinii* and *P. luminescens* at 20, 25 and 30 °C.

MATERIALS AND METHODS

Sources of the bacteria

The two entomopathogenic bacteria *X. boveinii* and *P. luminescens* were isolated from *S. feltiae* and *H. bacteriophora* respectively.

Methods for staining and observations

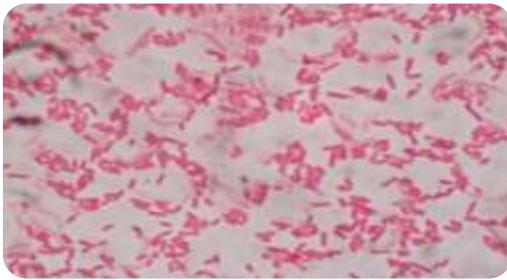
The method depends on the ability of some bacterial cells to retain the crystal violet-iodine complex when treated with alcohol. Such bacteria are designated as

gram-positive. Bacteria which are decolorised by alcohol after being stained with crystal violet and treated with iodine are designated as gram-negative. The method used was that described by Johnston and Booth (1983). Following were the steps of the protocol.

1. For micro-organisms growing on solid medium in a test tube, a loopful of tap water was placed on a microscope slide at the point where the smear was to be made.
2. The loop was flamed, the test-tube plug was removed, the mouth of tube was flamed, the loop was inserted and scraped the culture and removed, the mouth of tube was flamed again and the plug replaced.
3. The microbial growth collected on the loop was spread in the drop of water to give a thin smear which was then dried by gently warming and, when dry, fixed

by momentarily heating in the flame.

4. The slide was allowed to cool and placed on a staining rack.
5. The slide was then placed in crystal violet solution and stained for 1 minute.
6. The stain was then poured off and excess was washed away with iodine solution ensuring that the back of the slide was free strain. The smear was covered with iodine for 1 min.
7. The iodine was washed off with 95% alcohol repeatedly until the washing was pale violet colour.
8. The slide was then washed at once with water.
9. The smear was then counterstained with dilute safranin (0.5%) for 30 seconds.
10. Finally it was washed with water, blotted dry and then examined at x100 under oil immersion (Figure 1).



Xenorhabdus bovienii



Photorhabdus luminescens

Figure 1: Gram-negative staining of the bacteria of *Xenorhabdus bovienii* and *Photorhabdus luminescens*.

Extraction of DNA

For the extraction of DNA from *X. bovienii* and *P. luminescens* the following protocols were used (Rice, 2010).

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Sample collection and handling

For the extraction of DNA, single colonies grown on NBTA and cultures grown in nutrient broth (NB) were used.

Cell lysis

1. A 500 µl cell suspension from an overnight culture containing approximately 1-2 billion cells was added to a 1.5 ml microfuge tube on ice.
2. The tube was centrifuged at 13,000 rpm for 5 seconds, then, as much supernatant as possible was carefully removed with a pipette so as not to disturb the pellet of cells in the base of the tube.

3. 300 µl cell lysis solution was added and agitated with the pipette until cells were suspended.
4. The sample was incubated in hot water (80 °C) in an electric water bath for 5 minutes to lyse cells.

RNase treatment

1. 1.5 µl RNase A solution was added to the cell lysate.
2. The sample was mixed by inverting the tubes 25 times and incubated in water at 37 °C for 30 minutes in an electric water bath.

Protein precipitation

1. The sample was cooled on ice until it had decreased to room temperature.
2. 100 µl of protein precipitation solution was added to the cell lysate.
3. The tube was vortexed vigorously at high speed for 20 seconds to mix the protein precipitation solution uniformly with the cell lysate; it was then put on ice for 3-5 minutes.

- The tube was centrifuged at 13,000 rpm for 3 minutes by which time the precipitated proteins formed a tight pellet.

DNA precipitation

- The supernatant containing the DNA (leaving behind the precipitated protein pellet) was poured into a clean 1.5 ml microfuge tube containing 300 μ l 100% isopropanol (2-propanol).
- The sample was mixed by inverting gently 50 times.
- The tube was then centrifuged at 13,000 rpm for 1 minute; the DNA was visible as a small white pellet.
- The supernatant was discarded and the tube drained on clean absorbent paper, 300 μ l 70% ethanol was added and the tube inverted several times to wash the DNA.
- The tube was centrifuged at 13,000 rpm for 1 minute and the ethanol was carefully poured off.
- The tube was drained on clean absorbent paper and allowed to air dry for 10 minutes.

DNA hydration

- 50 μ l DNA hydration solution was added.
- The DNA was rehydrated by incubating sample for 1 hour at 65 °C.
- The DNA was stored in a fridge for further processing.

Polymerase chain reaction (PCR)

Preparation of mastermix

The Mastermix was prepared using the protocol provided by Dr Robert Jackson School of Biological Sciences, University of Reading (Personal Communication) using the following reagents.

Reagents	Component (μ l)
10xBuffer	5
50 mM MgCl ₂	2.5
100mM dNTPs	1
Primer 100 μ M	1 of each primer
Bio X Act	1
H ₂ O	38.5

The buffer, MgCl₂, and Taq were products of Promega, Epsilon and 154 IR were obtained from PUREGENE® whilst the dNTPs was product of BIOLINE. Glass deionised water was used. Water was the first to be put into the tubes and then the buffer, MgCl₂, dNTPs, Epsilon and 154IR were added. The mixture was spun at 2000 rpm for 1 minute. Taq was added and mixed on a whirl

mixer for 30 seconds.

Then 50 μ l of Mastermix was put in two PCR tubes; 1 μ l each of DNA materials, from *X. bovienii* and *P. luminescens* was added separately to the two PCR tubes containing the Mastermix.

PCR conditions

The PCR was run on a MJ DNA engine PTC 200 under the following conditions. (Tailliez et al.) for 1 cycle {70 °C for 1 minutes, 95 °C for 30 seconds, 55 °C 30 seconds}{Boemare, 2002; Fischer-Le Saux et al., 1998} for 30 cycles {70 °C for 10 minutes, 4 °C hold}

This implies that the DNA was hot started at 95 °C for 5 minutes. This was followed by heating at 95 °C for 5 minutes, 55 °C for 1 minute. This second section/cycle was repeated 30 times. The DNA engine was run at 70 °C for 10 minutes, 4 °C hold. Finally, PCR products were kept in a fridge at 4 °C.

Preparation of gel

Agarose (0.5 g) was added to 50 ml 1X TRIS BORATE-EDTA (TBE) to make 1% w/v. The mixture was melted using a microwave and heated to about 96 °C whilst continuously mixing/whirling in the conical flask to ensure that all the agarose was completely dissolved. The mixture was allowed to cool and 5 μ l of ethidium bromide was added to stain the DNA. The mixture was poured into a gel mould (gel tray), and left for 30 minutes to set with a well-forming comb.

The gel with wells was immersed in the tank and filled with TBE buffer to cover the wells. Loading buffer of 5 μ l and 5 μ l of PCR reagent were mixed thoroughly on a sealing film (Nescofilm) and pipetted into the wells along with Hyperladder 1. The gel was run for 30 minutes at 120 volts.

Visualization of gel in transilluminator

The gel was visualised using an ultraviolet transilluminator for formation of DNA bands of the two bacteria. The print of the bands was taken; the clear DNA bands were observed (Figure 2).

DNA sequencing and alingment

PCR products were cleaned using QiaQuick columns (Qiagen, Crawley, UK) as described by the manufacturers. Cleaned PCR products were sequenced using the forward primer epsilon using the Big Dye terminator sequencing kit (ABI, Warrington, UK) using conditions recommended by the manufacturers. Completed sequencing reactions were cleaned using ethanol precipitation and were sequenced at the BIOLINE Centre in the University of Reading, Plant

Science Building. Cleaned, unambiguous sequences were analysed in three ways. The sequence were checked using the Basic Local Alignment Search Tool (BLAST) facility at NCBI (National Centre for Biotechnology Information) (<http://www.ncbi.nlm.nih.gov/> September, 2009), using the identification tool on the Ribosome Database project (<http://rdp.cme.msu.edu/index.jsp>

September, 2009) (Maidak et al., 1994) by neighbour-joining analysis using Jukes and Cantors Algorithm after alignment of the sequences in ARB-from Latin word "arbour" meaning tree (Ludwig et al., 2004) and analysis using neighbour joining in Phylogenetic Analysis Using Parsimony (PAUP) (Swofford, 1998). Bootstrap analysis was used to evaluate the dendrogram.

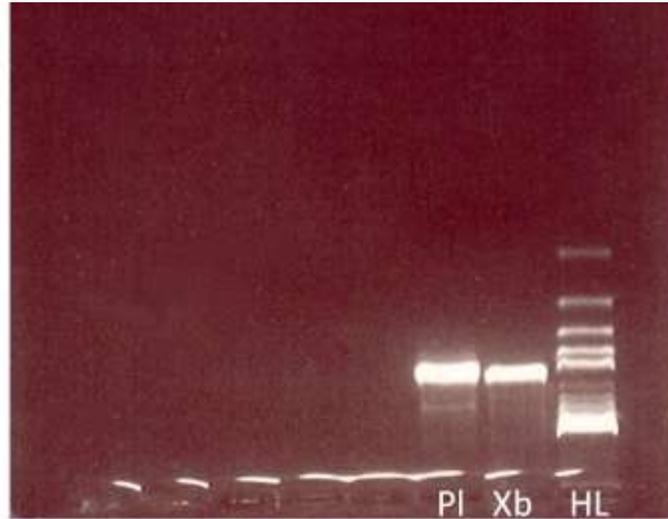


Figure 2: DNA band of *Photorhabdus luminescens* (PI) *Xenorhabdus bovienii* (Xb) and HypoLadder (HL).

Growth of *Xenorhabdus bovienii* and *Photorhabdus luminescens* at 20, 25 and 30 °C

Pure colonies of *X. bovienii* (D 78007) and *P. luminescens* (Z 76751) were multiplied in nutrient broth for 24 hours in a mechanical shaker at 150 rpm. The concentration of both the bacteria was calibrated and diluted to be equal using a spectrophotometer on the 600 nm scale.

The two bacteria with equal volumes of 100 ml were kept separately in 250 ml conical flasks and placed in a mechanical shaker with a temperature of 20 °C. On an hourly basis, 1 ml aliquot of the bacteria was sampled using a sterilized pipette and the optical density was determined on the spectrophotometer (on the 600 nm scale). The experiment was repeated at 25 °C and 30 °C. The growths of bacteria curves were determined using the statistical Regression analysis (exponential (or) asymptotic model).

RESULTS

From the characterisation, the partial 16S gene sequences obtained for the two bacteria were subjected to blast-bootstrap analysis to obtain the phylogenetic tree.

From the phylogenetic tree (Figure 3), the two species of bacteria were found to belong to the genera *Xenorhabdus*

and *Photorhabdus*. The two bacteria were compared with *X. japonica* NR027194, *X. nematophila*, D78006 *X. poinarii* DQ211703, *X. beddingii* AY278675 and *X. kozodoii* Eu 190977, the results are shown in Table 1.

In assessing the similarity of *X. bovienii* with five other *Xenorhabdus* spp., it was found to be 96% similar to *X. nematophila* and *X. japonica* with Accession Numbers D78006 and NR027194 respectively. The *X. bovienii* was closer to *X. beddingii* 95% and *X. kazodoii* and 97% similarity to *X. poinarii*. The results showed that at 20 °C, both bacteria *X. bovienii* and *P. luminescens* increased to a log concentration of 7.5 after 6 hours (Figure 4). They then increased slightly up to 15 hours. At 25 °C *X. bovienii* had a concentration of 6.1 after 1 hour but only increased slowly to 6.6 after 15 hours. However, *P. luminescens* started with 5.6 after 1 hour but increased smoothly to 7.5 after 15 hours. *X. bovienii* and *P. luminescens* at 30 °C had a concentration of 6.2 after 1 hour.

P. luminescens increased more rapidly than *X. bovienii* at first but after 8 hours, *X. bovienii* increased more than *P. luminescens* up to 15 hours. (Figure 4) The interactions between three different temperatures and time hours was highly significant ($P < 0.001$).

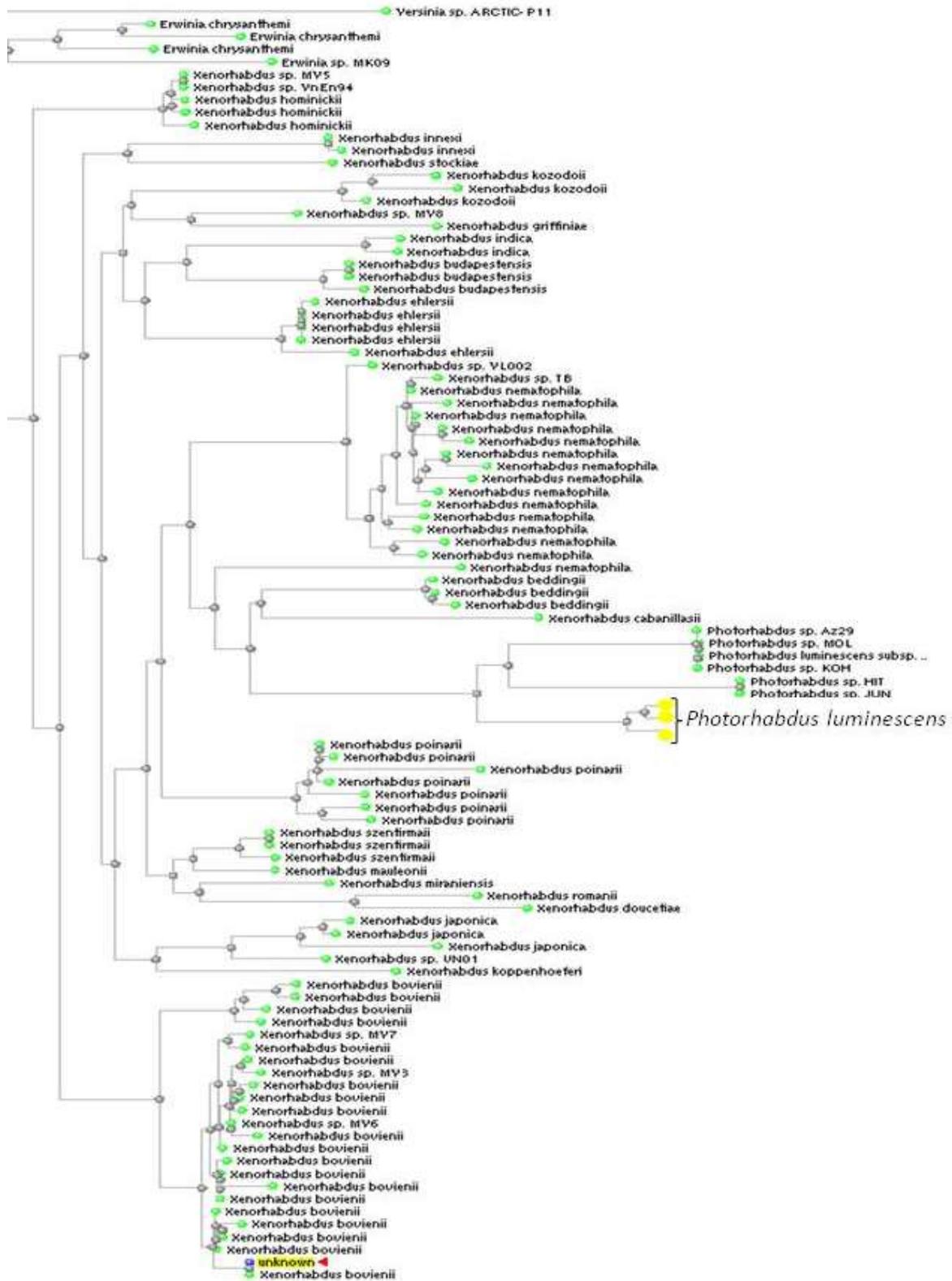


Figure 3: Phylogenetic analysis of partial 16S rRNA gene sequence from entomopathogenic bacteria isolated from nematodes and cultured isolates. Tree was derived after neighbour joining analysis of 425 basepairs using a Juke-cantor distance calculation. Bootstrap values (100 replicates, > 50%) are given at nodes.

Table 1: Comparison of percent similarity of *X. bovienii* and *P. luminescens* with *X. japonica*, *X. nematophila*, *X. kozodoii*, *X. poinarii* and *X. beddingii*.

Bacteria	Similarity (%)				
	<i>X. japonica</i> NR027194	<i>X. nematophila</i> D78006	<i>X. poinarii</i> DQ211703	<i>X. beddingii</i> AY278675	<i>X. kazodoii</i> Eu190977
<i>X. bovienii</i>	96 %	96 %	97 %	95 %	95 %
<i>P. luminescens</i>	96 %	96 %	97 %	95 %	95 %

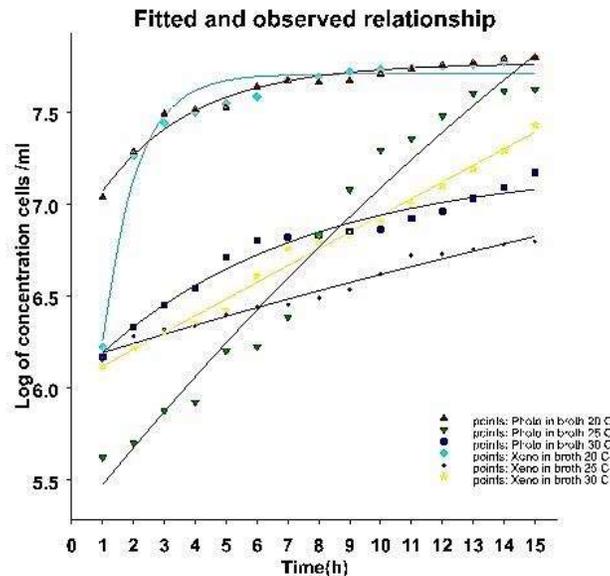


Figure 4: Concentration of cells as a function of time from inoculation during the exponential phase growth of cells of *Xenorhabdus bovienii* and *Photorhabdus luminescens* (Fitted and observed relationship) at different temperatures. Values shown are means of three replicates.

DISCUSSION

X. bovienii and *P. luminescens* grew and reproduced at 20, 25 and 30 °C. It is known that *X. bovienii* grows at temperatures up to 32 °C and some strains grew at 5 °C (Boemare, 2002; Boemare and Akhurst, 1988; Fischer-Le Saux et al., 1998). *X. bovienii* is associated with several species of steinernematid nematodes (*S. feltiae*, *S. intermedium*, *S. kraussei*, *S. affine*) from temperate regions (Boemare and Akhurst, 1988; Fischer-Le Saux et al., 1998). However, interestingly, the optimum growth temperature of *Photorhabdus* is usually 28 °C; but for some strains the upper threshold for growth (in nutrient broth) occurs at 35-39 °C (Boemare and Akhurst, 1988) suggesting that the *Photorhabdus*/*P. luminescens* complex is more effective in warmer soils and may be less effective than the *S. feltiae*/*X. bovienii* complex in British (temperate) soils.

From the molecular identification, *Xenorhabdus* sp. (D78007) was found to be closely related to *X. japonica*. Boemare (2002) reported that strains of *X. japonica* grew until 35 °C. It is possible that *Xenorhabdus* sp. (D78007) can also grow up to or beyond 35 °C.

The ability of both bacteria to grow from 20 to 30 °C makes them suitable candidates for the control of insect pests in the tropics (which was the rationale for this experiment). However, there have been observations that the *X. nematophila*-*Steinernema* complex is more cold tolerant than the *Photorhabdus*-*Heterorhabditis* complex (Blackshaw and Newell, 1987; Molyneux, 1986), and may also support the findings that *X. bovienii* tolerates lower growth temperatures better than *P. luminescens* (Clarke and Dowds, 1994).

From the foregoing results, the *Xenorhabdus* sp. and *Photorhabdus* sp. under study showed high similarity

with the various species of these genera. Thomas and Poinar (1979) and more recently, Sicard et al. (2003), affirmed that *Steinernema* spp. are symbiotically associated with Enterobacteriaceae of the genus *Xenorhabdus*. The bacterium *Xenorhabdus* obtained from *S. feltiae* was found to be *Xenorhabdus bovienii*. According to Boemare (2002), *X. bovienii* is only found associated with *S. feltiae*. *X. bovienii* shared 95-97% homology values with several strains of the *Xenorhabdus*. Sicard et al. (2004a); Sicard et al. (2004b) observed that only symbionts belonging to *X. bovienii* were transmitted by *S. feltiae*.

Cabanillas et al. (1994) reported that the mutualistic bacterium associated with *S. feltiae* and *H. bacteriophora* was isolated on nutrient agar. The primary bacterium was characterized by its adsorption of bromothymol blue from NBTA. From this study, these characteristics do not differ from the description of *X. bovienii*, but further studies need to be conducted.

AUTHORS' CONTRIBUTION

AMR designed the study, executed experimental work, collected data, AMR and RKR analyzed the data, AMR wrote the manuscript, RKR and MB assisted in writing the manuscript, AMR and MB proofread the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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