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Molecular Characterization and Antibacterial Activities of *Photorhabdus* and *Xenorhabdus* from Mizoram, North-East India

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Abstract

***Photorhabdus* and *Xenorhabdus* are the bacterial symbionts of insect pathogenic nematodes, *Heterorhabditis* and *Steinernema*, respectively. This study aims to characterize the bacterial symbionts from Mizoram, North-east India and to evaluate their antibacterial potential. The bacterial isolates were characterized using recA and gyrB gene regions. The ethyl acetate extract of bacterial isolates was tested against pathogenic bacterial strains, viz. *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 10145), and *Bacillus subtilis* (ATCC 11774) using disk diffusion method. Analysis of recA and gyrB genes revealed that the *Photorhabdus* isolates were *P. hindustanensis*, and *P. namnaonensis*. This study constitutes the first documentation of *P. namnaonensis* from India. The two isolated *Xenorhabdus* belong to *X. vietnamensis* and *X. stockiae*. The ethyl acetate extracts of the studied bacteria suppressed the development of all the microorganisms tested. Based on MIC and MBC values, the highest activity was exhibited by TS (*P. hindustanensis*) and TD (*P. namnaonensis*) isolates against *P. aeruginosa* and *K. pneumoniae* respectively. The lowest inhibitory activity was observed on both *Xenorhabdus* isolates (RF and PTS) against *B. subtilis.* This study focuses on the existence and identification of symbiotic bacteria from Mizoram, an Indo-Burma biodiversity hotspot region, and details their activity against different pathogenic bacteria. Since these metabolites could be potent antibiotics, further research is required to better understand the genetic information, chemical composition, and method of action against other microorganisms.**

Keywords: Antibiotic, *Photorhabdus*, *Xenorhabdus*, gyrB, recA

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INTRODUCTION

Microbial resistance to classical antibiotics has long been a serious health concern worldwide. Though resistance occurs naturally, the overuse or misuse of the existing antibiotic drugs in a variety of formats exerted selective pressure on certain microorganisms. This, in turn, develops resistance against antibiotics resulting in less or no effectiveness to treat various diseases.[1] Furthermore, widespread antibiotic use and self-medication by farmers and patients, respectively, as well as hospital infection exposure, have accelerated the growth of multidrug-resistant (MDR) bacteria globally.[2–4] The widespread antibiotic resistance was reported globally and declared a pandemic.[5] Recently, the World Health Organization (WHO) has listed a group of pathogen strains, including *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. These strains are becoming more virulent and resist multidrug; therefore, effective antibiotics are urgently required for treatment.[2,6] Also, these organisms can avoid the effects of the current antimicrobial medications and are also responsible for the majority of nosocomial infections[-],[2,7] thereby consequently linked to the highest risk of mortality and morbidity, which raises healthcare expenses.[8] Thus, an increase in the development of resistance in clinically important bacterial strains has led to the demand for the discovery and development of effective antibiotics.

Photorhabdus and *Xenorhabdus* are gram-negative bacteria under the family Morganellaceae. They formed a mutual relation with insect pathogenic nematodes viz *Heterorhabditis* and *Steinernema*, respectively.[9] They have a cosmopolitan distribution (except Antarctica) and are highly effective at controlling a variety of insect pests.[10] Altogether, *Photorhabdus* has 28 recognized taxa, including 22 species, six of which are further classified into subspecies.[11] So far, 28 taxa of *Xenorhabdus* associated with the nematodes have been identified including two recently identified subspecies.[12] When the mutualistic association infects the insect larvae, the symbiotic bacteria produce broad-spectrum compounds which are lethal to the infected larvae, including activity against bacteria, fungi and parasites.[13] Meanwhile, several compounds produced by bacterial symbionts are known to protect the insect cadaver microenvironment due to their antimicrobial, nematicidal, acaricidal and insecticidal activity.[14-16] The bacteria multiplied within the insect host, the nematodes ingest the bacteria and consume the cadaver for growth and reproduction.[17] When the food resources are exhausted, the emergence of the infective juveniles (IJs) from the host insect occurred and IJs eventually seek a new host.[18]

The secondary metabolites of *Photorhabdus* spp. and *Xenorhabdus* spp. including methanol and ethyl acetate extracted bioactive compounds from the fermented culture media were found to be effective in controlling various pathogenic bacteria.[19-21] and fungi [14, 22-24] to a great extent. In addition to antibacterial and antifungal, the bioactive compounds have been claimed to be effective against insects.[13,25,26] Therefore, these bioactive compounds might be the solution to the novel antimicrobial compounds and could be used to overcome the limitations of effective antibiotics in combating certain disease-causing microbes.

The present study aims to characterize the insect pathogenic nematode-associated bacteria from Mizoram, North-east India using two housekeeping genes, *viz.* recA and gyrB gene regions, and to further assess the antibacterial activity of the bacterial symbionts against four pathogenic bacteria viz. *E. coli* (ATCC 10536), *K. pneumoniae* (ATCC 10031), *P. aeruginosa* (ATCC 10145), and *B. subtilis* (ATCC 11774) using the disk diffusion method.

MATERIALS AND METHODS

Isolation and identification of bacterial symbionts

The symbiotic bacteria were isolated from entomopathogenic nematodes (EPNs) viz. *Heterorhabditis indica* (location: 23.740N 92.952E), *H. baujardi* (location: 22.350N 93.060E), *Steinernema sangi* (location: 23.370N 93.161E) and *S. surkhetense* (location: 22.960N 92.612E) which were randomly collected from four different localities of Mizoram, North-East India. The freshly emerged infective juveniles (IJs) of

insect pathogenic nematodes were macerated for isolation of bacterial symbionts.[27] Prior to the maceration process, the IJs were washed with 10% sodium hypochlorite (w/v) to prevent external tegument contamination. The IJs were further crushed in sterile PBS buffer using a micro-pestle. A volume of 100 µl was inoculated on nutrient agar with 0.0025% bromothymol blue and 0.004% triphenyl tetrazolium chloride (NBTA).[27,28] followed by incubation for 48 h at 28R"C.

Bacterial cells from overnight nutrient broth culture were harvested and Phenol Chloroform Isoamyl-alcohol (PCI) method was used for genomic DNA extraction.[29] For phylogenetic analysis, a set of primer- recA1 F (5'-GCTATTGATGAAAATAAACA-3') and recA2 R (5'-RATTTTRTCWCCRTTRTAGCT-3') was used for the amplification of recombinase A gene.[30] In addition, another set of primer- 1200FgyrB (5'-GATAACTCTTATAAAGTTTCCG-3') and 1200RgyrB (5'- CGGGTTGTATTCGTCACGGCC-3') was used for amplification of gyrase B gene.[30] The PCR conditions applied for recA were 5 min at 94°C for denaturation followed by 30 cycles for 1 min at 94°C, 1 min at 55°C for annealing and 1 min at 72°C for extension followed by 5 min at 72°C. For gyrB, the PCR conditions were set as follows: 5 min at 94°C for denaturation followed by 30 cycles for 1 min at 94°C, 45 s at 58°C for annealing and 2 min at 72°C for extension followed by 7 min at 72°C. The PCR products were directly sequenced in a forward direction.

Sequence alignment and analysis

Sequence editing was performed using FinchTV 1.4.0 (http://www.geospiza.com) and alignment was done using clustalW (MEGA X).[31] Sequences were compared with the nearest matches available species from GenBank using the BLASTN algorithm. For phylogenetic analysis, 29 and 30 available taxa of *Xenorhabdus* and *Photorhabdus* respectively including an outgroup species, *E. coli* K-12 was recovered from NCBI GenBank.

To calculate genetic distance, pairwise sequence comparisons using the Kimura 2-Parameter approach under the Gamma distribution were used. The maximum likelihood tree (ML) was generated following the lowest BIC score (K2P with G + I sites) and branches statistically supported by a replicate of 1000 bootstraps. The generated sequences were deposited to NCBI GenBank (Accession number ON314147-ON314170).

In addition, the nucleotide sequences of recA and gyrB genes were concatenated, aligned and the ML tree was constructed separately for *Photorhabdus* and *Xenorhabdus* spp with the available type strain retrieved from NCBI GenBank.

Preparation of pathogenic bacteria.

Four strains of pathogenic bacteria, viz. *E. coli* (ATCC 10536), *K. pneumoniae* (ATCC 10031), *P. aeruginosa* (ATCC 10145), and *B. subtilis* (ATCC 11774) were maintained in Research and Instrumentation Centre, Pachhunga University College, Aizawl. A bacterial colony was transferred in nutrient broth followed by incubation at 30°C for a duration of 24 h. The overnight grown culture was adjusted to 0.5 McFarland standard for further antibacterial assay.

Preparation of bacterial extract

The extraction of bacterial metabolites and preparation of stock concentrations were prepared according to Muangpat et al.[21] with a slight modification. The whole-cell suspension of the bacterial isolates was used for preliminary screening of antibacterial activity. A 50 µl of the cell suspension was incorporated into the agar well containing the spread pathogenic strain and kept at 30°C for 24 h. A void zone around the well was read as an inhibition zone. For all the bacterial isolates, a colony was shifted to a 1000 ml sterile nutrient broth, stored at 28°C in a shaker incubator for 48 h and subsequently transferred to 2000 ml of separating funnel. To extract the crude compound, the same volume of whole-cell suspension and ethyl acetate were mixed by inverting the funnel and laid at room temperature for 24 h. Further, ethyl acetate layer was collected, then evaporated using Rotavapor® R-100 System-Buchi, Switzerland. To obtain the maximum amount of crude extract, the extraction procedure was repeated twice.

A stock solution was made by solvating 500 mg of the condensed bacterial extract in 1 ml of DMSO. A unit of 10 µl from the stock solution was pipetted out and impregnated into 6 mm

paper disks. The paper disks were then placed on Mueller Hinton Agar (MHA) previously plated with the selected pathogenic strain followed by incubation at 30°C for a duration of 24 h. A caliper was used to measure the diameter of a clear zone (in mm). A drop of DMSO served as a negative control, whereas a standard ampicillin disk served as a positive control.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC of the bacterial extracts was performed following the microdilution method in a 96-well microtiter plate. The same volume of bacterial extracts and sterile Mueller Hinton Broth (MHB) was mixed in a well followed by two-fold serial dilutions. Then, each well was inoculated with 10 µl of microbial inoculum initially adjusted to 0.5 Mc Farland standard. After thorough mixing, plate incubation was performed at 30 °C for 24 h. For control, a DMSO mixture and nutrient broth with inoculum and the same mixture without inoculum were used. The MIC was determined as minimum bacterial extract concentration along with a clear well as detected by the unaided eye.

For MBC, 10 µl of the extract dilution representing the MIC along with two more concentrated diluted wells were streaked on MHA to observe visible growth. The plates incubation was done at 30 °C for 24 h and growth was observed corresponding to different concentrations. The MBC value was determined as minimum bacterial extract concentration without observable growth on MHA.

Statistical data analysis

The zone of inhibition size (in diameter) is given as Mean ± Standard Error of Mean (SEM). One-way ANOVA was carried out to determine a variation of inhibition exhibited by the bacterial isolates (at the level of $P \leq 0.05$) against the selected pathogenic bacteria.

RESULTS

Characterization of the bacteria

The two *Photorhabdus* isolates TS and TD were isolated from *H. indica* and *H. baujardi*, respectively, while the two *Xenorhabdus* isolates, RF and PTS were isolated from *S. sangi* and *S. surkhetense*, respectively.

The total length of the recA sequence developed is 860 bp. The two isolates, TS and TD, consistently exhibited 2.2% K2P distance gap (98.1% similarity) between them. Further, based on the analysis of the developed gene region with closely related type species from the GenBank, the *Photorhabdus* isolates (TS) exhibited a closer relationship with *P. hindustanensis* (PUWT01) (99.53% similarity with 0.8% K2P distance). Another *Photorhabdus* isolate (TD) showed 98.59% (1.6% K2P distance) similarity with *P. namnaonensis* (LOIC01). Further analysis showed that the *Photorhabdus* (TS) isolate exhibited 99.41% (0.9% K2P distance), 98.24% (2% K2P distance) and 98.13% (2% K2P distance) similarity with *P. akhurstii* subsp. *akhurstii* (RCWE01), *P. hainanensis* (RCWD01) and *P. akhurstii* subsp. *bharatensis* (RCWU01) respectively, whereas, the *Photorhabdus* isolate (TD) exhibited 98% (2.2% K2P distance), 97.83% (2.3% K2P distance), and 96.9% (3.0% K2P distance) similarity with *P. hindustanensis* (PUWT01), *P. akhurstii* subsp. *akhurstii* (RCWE01) and *P. hainanensis* (RCWD01), respectively. Simultaneously, the two *Xenorhabdus* isolates, RF and PTS exhibited highest similarity with *X. vietnamensis* (FJ823401) (99.69% similarity with 0.3% K2P distance) and *X. stockiae* (KX826948) (99.4% similarity with 0.6% K2P distance), respectively. Further, the isolates, RF showed 95.5% similarity with *X. japonica* (FJ823400) and PTS isolates showed 93.21% similarity with *X. innexi* (FJ823423).

The total length of the gyrB sequence developed is 1050 bp. The two *Photorhabdus* isolates, TS and TD showed 96.88% similarity (3.2% K2P distance) between them. Further analysis of the isolates and comparison with the type species on NCBI GenBank showed that the *Photorhabdus* isolates (TS) exhibited 99.43% (0.5% K2P distance) similarity with *P. hindustanensis* (PUWT01), whereas, *Photorhabdus* (TD) isolates exhibited the highest similarity with *P. hainanensis* (RCWD01) (98.30% similarity with 1.7% K2P distance. Comparisons with other closely related species show that *Photorhabdus* isolates (TS) exhibited 97.83% (2.2% K2P distance), 96.60% (3.5% K2P distance), 97.17% (2.9% K2P distance) similarity

Table 1. Sequences analysed in this study

Strain	Species	Nematode Host	NCBI Accession No. (recA)	NCBI Accession No. (gyrB)	Country	Ref.
TS1	*P. hindustanensis*	*H. indica*	ON314156	ON314147	Mizoram, India	This study
TS2	*P. hindustanensis*	*H. indica*	ON314157	ON314148	Mizoram, India	This study
TS3	*P. hindustanensis*	*H. indica*	ON314158	ON314149	Mizoram, India	This study
TD1	*P. namnaonensis*	*H. baujardi*	ON314159	ON314150	Mizoram, India	This study
TD2	*P. namnaonensis*	*H. baujardi*	ON314160	ON314151	Mizoram, India	This study
TD3	*P. namnaonensis*	*H. baujardi*	ON314161	ON314152	Mizoram, India	This study
RF1	*X. vietnamensis*	*S. sangi*	ON314162	ON314168	Mizoram, India	This study
RF2	*X. vietnamensis*	*S. sangi*	ON314163	ON314169	Mizoram, India	This study
RF3	*X. vietnamensis*	*S. sangi*	ON314164	ON314170	Mizoram, India	This study
PTS1	*X. stockiae*	*S. surkhetense*	ON314165	ON314153	Mizoram, India	This study
PTS2	*X. stockiae*	*S. surkhetense*	ON314166	ON314154	Mizoram, India	This study
PTS3	*X. stockiae*	*S. surkhetense*	ON314167	ON314155	Mizoram, India	This study
MEX47 -22^{T}	*Photorhabdus P. luminescens. subsp*	*H. mexicana H. mexicana*	PUJX01	PUJX01	Mexico	43
Hb ^{T}	*P. luminescens* subsp. *luminescens*	*H. bacteriophora*	FMWJ01	FMWJ01	Australia	43
JAR^{T}	*P. luminescens* subsp. *venezuelensis*	*H. amazonensis*	JAPFFZ01	JAPFFZ01	Venezuela	11
C1^{T}	*P. khanii* subsp. *khanii*	*H. bacteriophora*	AYSJ01	AYSJ01	USA	43
MEX20 -17^{T}	*P. khanii* subsp. *guanajuatensis*	*H. atacamensis*	PUJY01	PUJY01	Mexico	43
TTO1^{T}	*P. laumondii* subsp. *laumondi*	*H. bacteriophora*	WSFH01	WSFH01	Australia	44
BOJ47^{T}	*P. laumondii* subsp. *clarkei*	*H. bacteriophora*	NSCI01	NSCI01	Iran	44
FRG04^{T}	*P. akhurstii* subsp. *akhurstii*	*H. indica*	RCWE01	RCWE01	Australia	45
H3^{T}	*P. akhurstii* subsp. *bharatensis*	*Heterorhabditis* sp.	PUWU01	PUWU01	India	45
SF41^{T}	*P. heterorhabditis* subsp. *heterorhabditis*	*H. zealandica*	RCWA01	RCWA01	South Africa	46
Q614^{T}	*P. heterorhabditis* subsp. *aluminescens*	*Heterorhabditis* sp.	JABBCS01	JABBCS01	Australia	46
9802892^{T}	*P. australis* subsp. *australis*	Clinical specimen	JONO01	JONO01	Australia	46
PB68.1^{T}	*P. australis* subsp. *thailandensis*	*H. indica*	LOMY01	LOMY01	Thailand	46

Table 1. Cont...

Strain	Species	Nematode Host	NCBI Accession No. (recA)	NCBI Accession No. (gyrB)	Country	Ref.
AM7^T	*P. noenieputensis*	*H. noenieputensis*	JQ424881	JQ424884	South Africa	44
C8404^T	*P. hainanensis*	*Heterorhabditis* sp.	RCWD01	RCWD01	China	44
PB45.5^T	*P. namnaonensis*	*H. baujardi*	LOIC01	LOIC01	Thailand	47
HG29^T	*P. caribbeanensis*	*H. bacteriophora*	RCWB01	RCWB01	Guadeloupe	44
H1^T	*P. hindustanensis*	*Heterorhabditis* sp.	PUWT01	PUWT01	India	45
DSM 23513^T	*P. kleinii*	*H. georgiana*	JAJAFY01	JAJAFY01	North America	44
DSM 15194^T	*P. kayaii*	*H. bacteriophora*	JAJAFZ01	JAJAFZ01	Turkey	44
LJ24-63^T	*P. bodei*	*H. beicherriana*	NSCM01	NSCM01	China	44
BA1^T	*P. aegyptia*	*H. indica*	JFGV01	JFGV01	Egypt	44
39-8^T						
PT1.1	*P. thracensis*	*H. bacteriophor*a	CP011104	JAGJJU01	Turkey	48, 44
XINach^T	*P. temperata*	*H. megidis*	JAJAFX01	AUXQ01	Russia	44, 49
M1021						
DSM 23271^T	*P. stackebrandtii*	*H. bacteriophora*	PUJV01	PUJV01	North America	44
T327^T	*P. tasmaniensis*	*H. zealandica*	PUJU01	PUJU01	Australia	44
UCH-936^T	*P. antumapuensis*	*H. atacamensis*	JAHZMK01	JAHZMK01	Chile	50
3107^T	*P. cinerea*	*H. downesi*	PUJW01	PUJW01	Hungary	44
3265-86^T	*P. asymbiotica*	Clinical specimen	RBLJ01	RBLJ01	USA	44
APURE^T	*P. aballayi*	*H. amazonensis*	JAPFCD01	JAPFCD01	Switzerland	11
VN01^T	*X. vietnamensis*	*S. sangi*	FJ823401	EU934514	Vietnam	30
DSM 16522^T	*X. japonica*	*S. kushidai*	FJ823400	EU934513	Japan	30
ID10 T	*X. griffiniae*	*S. hermaphroditum*	FJ823399	EU934525	Indonesia	30
DSM 16337^T	*X. ehlersii*	*S. serratum*	FJ823398	EU934524	China	30
FRM16^T	*X. doucetiae*	*S. diaprepesi*	FJ823402	EU934526	Martinique	30
PR06-A^T	*X. romanii*	*S. puertoricense*	FJ823403	EU934515	Puerto Rico	30
SaV^T	*X. kozodoii*	*S. arenarium*	FJ823404	EU934522	Russia	30
G6^T	*X. poinarii*	*S. glaseri*	FJ823409	EU934543	USA	30
SF87^T	*X. khoisanae*	*S. khoisanae*	AB685736	AB685735	South Africa	52
Q1^T	*X. miraniensis*	*Steinernema* sp.	FJ823414	EU934520	Australia	30
Q58^T	*X. beddingii*	*Steinernema* sp.	FJ823415	EU934516	Australia	30
KE01^T	*X. hominickii*	*S. karii*	FJ823410	EU934517	Kenya	30
USNJ01^T	*X. koppenhoeferi*	*S. scarabaei*	FJ823413	EU934532	USA	30
ATCC 19061^T	*X. nematophila*	*S. carpocapsae*	FN667742	FN667742	USA	53
DSM 16338^T	*X. szentirmaii*	*S. rarum*	FJ823416	EU934534	Argentina	30
VC01^T	*X. mauleonii*	Unknown	FJ823417	EU934533	St. Vincent	30
VP-2016b	*X. stockiae*	*S. surkhetense*	KX826948	KX826949	India	54
TH01^T	*X. stockiae*	*S. siamkayai*	FJ823425	EU934542	Thailand	30
DSM 16336^T	*X. innexi*	*S. scapterisci*	FJ823423	EU934540	Uruguay	30
DSM 16342^T	*X. budapestensis*	*S. bicornitum*	FJ823418	EU934535	Serbia	30
IMI 397775^T	*X. magdalenensis*	*S. australe*	FJ798401	JF798402	Chile	51

Table 1. Cont...

Strain	Species	Nematode Host	NCBI Accession No. (recA)	NCBI Accession No. (gyrB)	Country	Ref.
DSM 17382^T	*X. indica*	*S. thermophilum*	FJ823421	EU934538	India	30
USTX62^T	*X. cabanillasii*	*S. riobrave*	FJ823422	EU934537	USA	30
T228 T	*X. bovienii* subs *p. bovienii*	*S. feltiae*	JANAIF01	JANAIF01	Australia	12
XENO-1^T	*X. bovienii* subs *p. africana*	*S. africanum*	JAMGSK01	JAMGSK01	Africa	12
VLST	*X. lircayensis*	*S. unicornum*	JACOII01	JACOII01	Chile	55
30TX1^T	*X. thuongxuanensis*	*S. sangi*	KX602194	KY451961	Vietnam	56
DL20^T	*X. eapokensis*	*S. eapokense*	KX602188	KY451960	Vietnam	56
GDh7^T	*X. ishibashii*	*S. aciari*	AB630947	AB630948	Japan	57
K-12	*Escherichia coli*		NC_000913.3	NC_000913.3		Gen Bank

with *P. akhurstii* subsp. *akhurstii, P. akhurstii* subsp. *bharatensis* (RCWU01) and *P. hainanensis* (RCWD01) respectively, whereas, the *Photorhabdus* (TD) isolates showed 97.26% (2.8% K2P distance), 96.22% (2.8% K2P distance) and 96.88% (3.2% K2P distance) similarity with *P. namnaonensis* (LOIC01) and *P. akhurstii* subsp. *akhurstii* (RCWE01) and *P. hindustanensis* (PUWT01) respectively. Simultaneously, the *Xenorhabdus* isolates, RF and PTS, showed 99.88% and 99.15% (0.0-0.8% K2P distance) similarity with the database sequence of *X. vietnamensis* (EU934514) and *X. stockiae* (KX826949) respectively. Further, among the *Xenorhabdus* isolates, RF showed 95.48% similarity with *X. japonica* (EU934513) and PTS showed 93.97% similarity with *X. innexi.*

In addition, the ML tree constructed from the concatenation of the two nucleotide sequences (recA and gyrB genes) showed that the *Photorhabdus* isolates, TS and TD clustered cohesively with the type strain of *P. hindustanensis* H1^T (0.6% K2P distance) and *P. namnaonensis* PB45.5^T (2%) respectively. Also, the *Xenorhabdus* isolates, RF and PTS are closely related with the type strain of *X. vietnamensis* VN01^T (0.1% K2P distance) and *X. stockiae* TH01^T (3% K2P distance) respectively.

The maximum likelihood tree of bacterial symbionts and database sequences from NCBI GenBank are given in Table 1 and Figure 1-6.

Antibacterial activity

The extract of all bacterial isolates inhibited the growth of *E. coli* (ATCC 10536), *K. pneumoniae* (ATCC 10031), *P. aeruginosa* (ATCC 10145), and *B. subtilis* (ATCC 11774) within 24 h of incubation. (Figure 7). Additionally, from our study, the extracts of the bacterial isolates including standard ampicillin showed significant variations of growth inhibition against the selected pathogenic bacteria ($df=4,43$; $F=31.96$; $p<0.05$). However, no significant difference was observed in the activity of TS isolates and ampicillin($p>0.05$) while the other extracts showed a significant difference in

Table 2. Activity of bacterial extracts against pathogenic bacteria using disk diffusion method (Mean±SEM)

Bacterial strain	*E. coli* (ATCC 10536)	*K. pneumoniae* (ATCC 10031)	*P. aeruginosa* (ATCC 10145)	*B. subtilis* (ATCC 11774)	Standard (Ampicillin)
TS	15.33±0.33	16.33±0.33	14.33±0.33	13.67±0.33	15.67±0.33
TD	14±0.57	14.33±0.33	13.67±0.67	13.67±0.33	15.67±0.33
RF	13.33±0.33	13.67±0.33	13±0.57	13.33±0.33	15.33±0.33
PTS	10±0.57	11.67±0.3	10±0.57	10.67±0.33	16.67±0.33

activity when compared with standard ampicillin ($p>0.05$). Among the bacterial isolates, the extracts of TS and TD were found to be most potent against the pathogenic bacteria with a growth inhibition zone of 13.67– 16.33 mm diameter. The highest inhibition on the pathogenic bacteria was recorded with *K. pneumoniae* (ATCC 10031) where the bacterial extract of *Photorhabdus* isolates (TS) provided a clear inhibition zone of 16.33 ± 0.33 mm in diameter. In addition, the bacterial extract of *X. vietnamensis* inhibited the growth of all four tested bacterial strains, with a clear inhibition zone of 13–13.67 mm diameter. The extract of *X. stockiae* showed comparatively lower activity

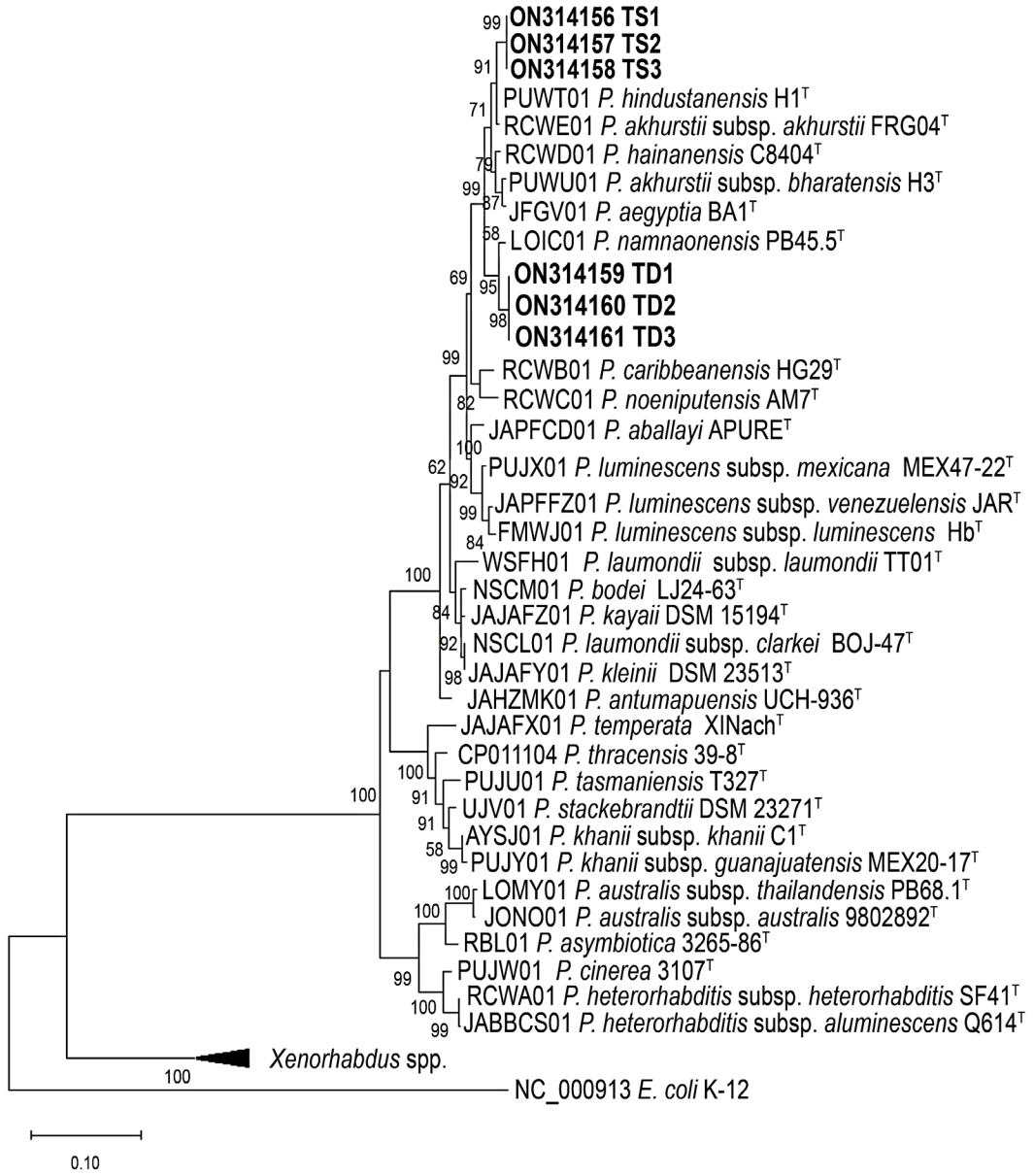

Figure 1. Maximum likelihood tree of *Photorhabdus* isolates inferred from recA gene. The numbers at the nodes correspond to the bootstrap support (1000 replicates, 50% or more). GenBank accession numbers and strain codes were given along with each species

against the pathogenic bacteria as compared to the other isolates, exhibiting a clear inhibition zone of 10–11.67 mm diameter against the studied bacterial strains. The Mean ± SEM of the inhibitory activity of the bacterial isolates against pathogenic bacteria was given in Table 2.

The MIC and MBC values of *Photorhabdus* and *Xenorhabdus* extracts against the morbific

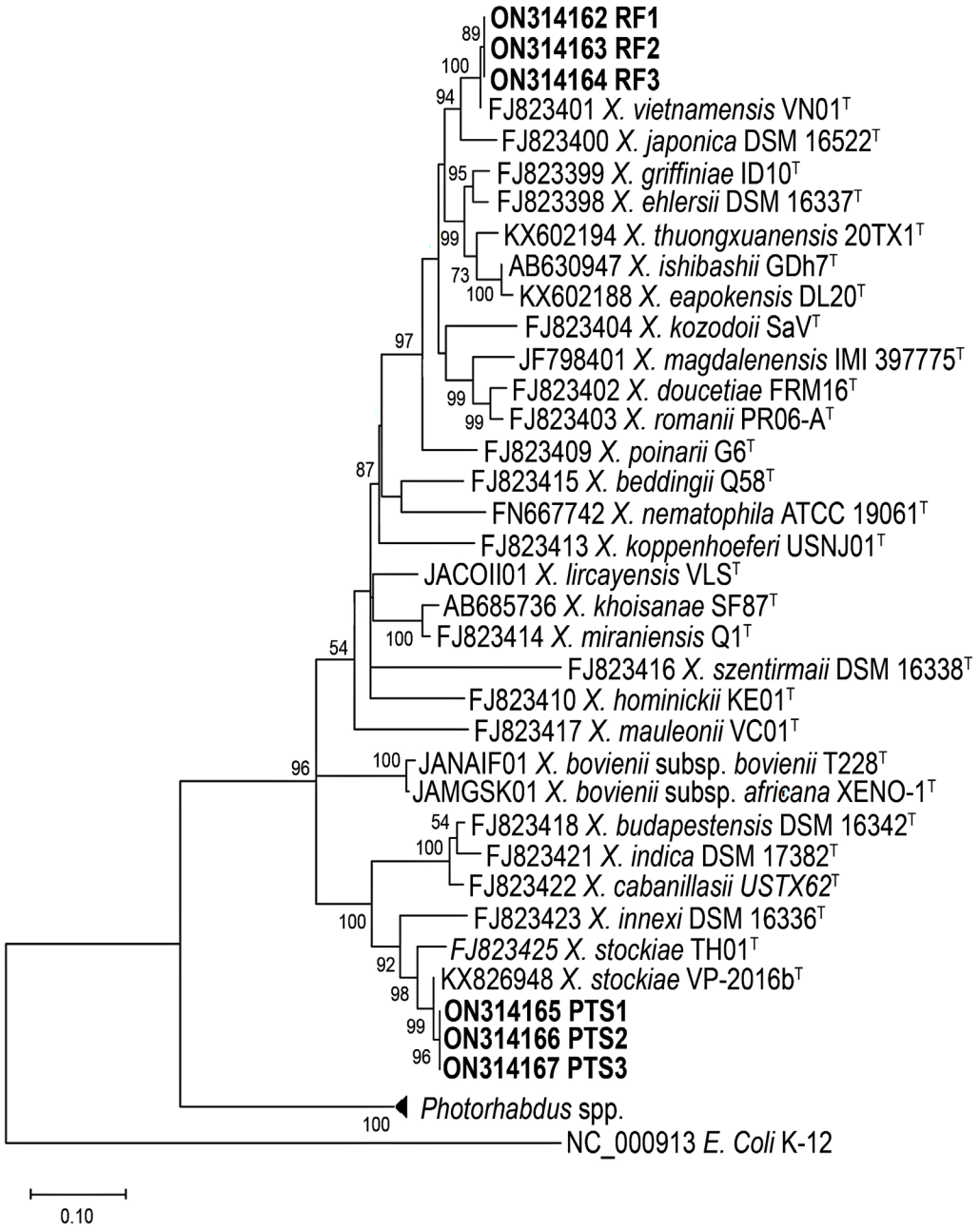

Figure 2. Maximum likelihood tree of *Xenorhabdus* isolates inferred from recA gene. The numbers at the nodes correspond to the bootstrap support (1000 replicates, 50% or more). GenBank accession numbers and strain codes were given along with each species

bacteria were given in Table 3. The MIC value of both *Photorhabdus* isolates ranges from 3.90–1.95 mg/ml and the MBC value range from 7.81–1.95 mg/ml. Furthermore, the *Photorhabdus* isolates, TS and TD exhibited the highest activity at the same MIC and MBC value (1.95 mg/ml) against *P. aeruginosa* (ATCC 10145) and *K. pneumoniae* (ATCC 10031), respectively. In addition, the lowest inhibitory activity was observed with *Photorhabdus* isolates (TS and TD) against *B.*

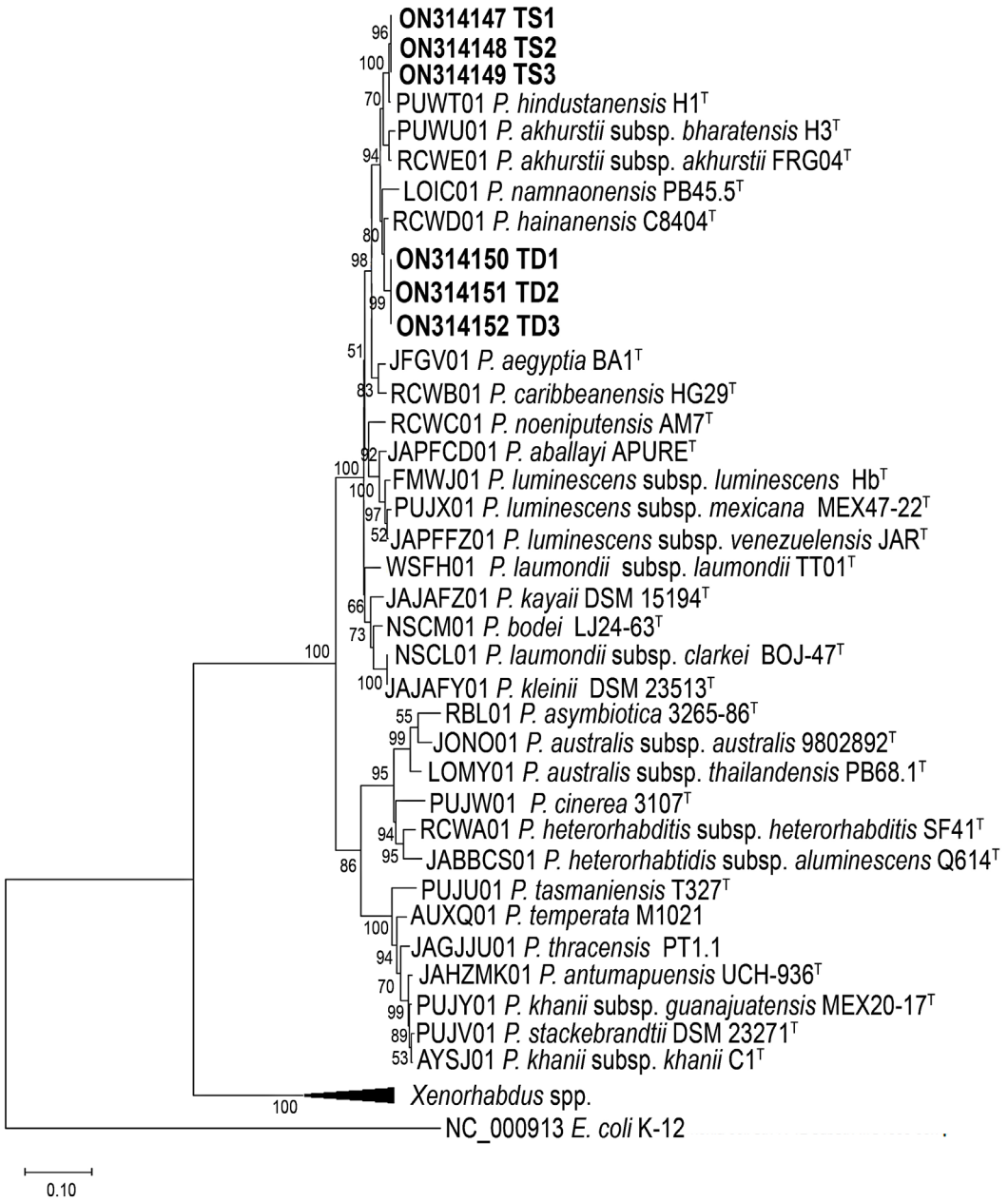

Figure 3. Maximum likelihood tree of *Photorhabdus* isolates inferred from gyrB gene. The numbers at the nodes correspond to the bootstrap support (1000 replicates, 50% or more). GenBank accession numbers and strain codes were given along with each species

subtilis at the MIC and MBC values of 3.90 mg/ml and 7.81 mg/ml, respectively.

In the case of *Xenorhabdus* isolates, both *X. vietnamensis* and *X. stockiae* show MIC

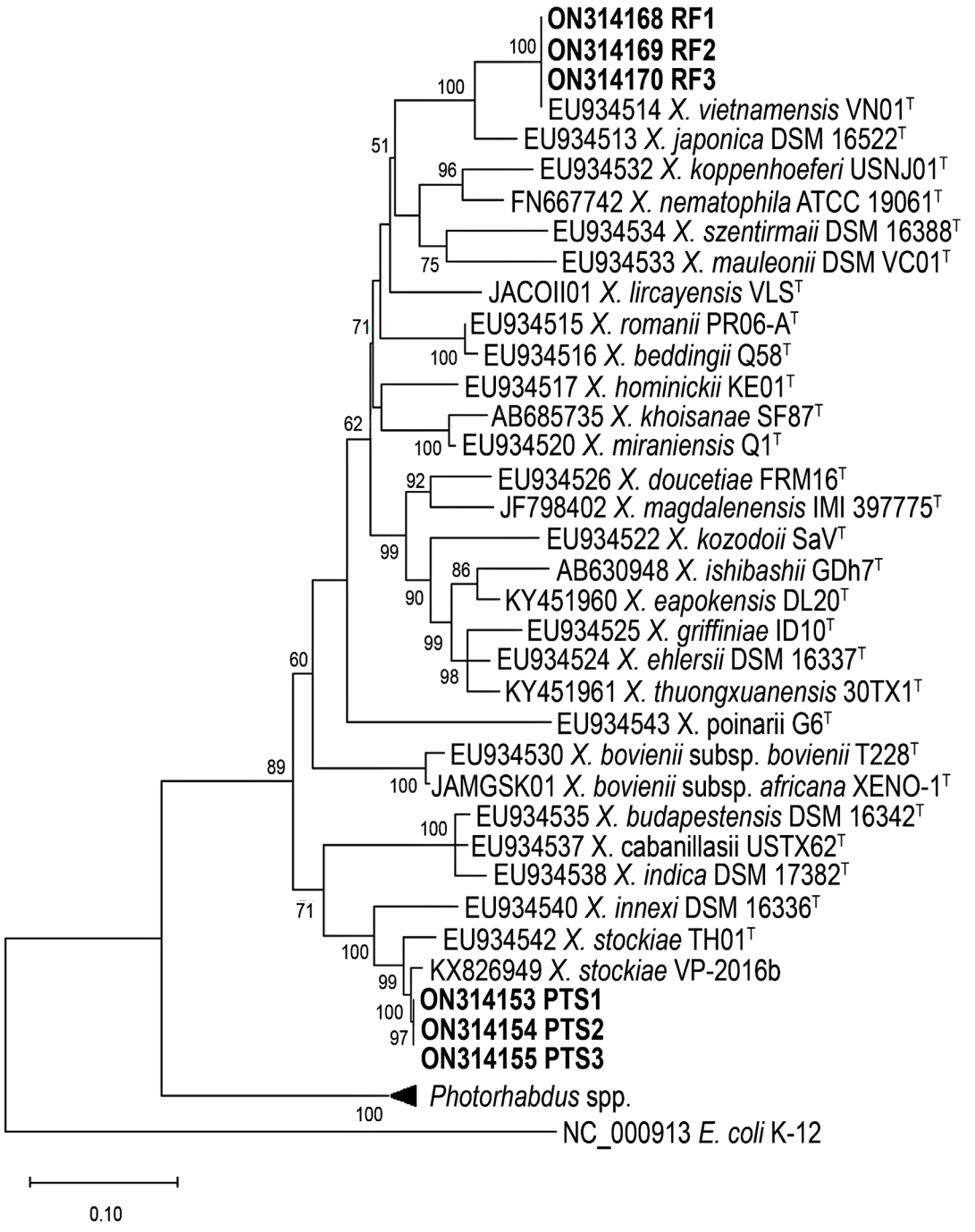

Figure 4. Maximum likelihood tree of *Xenorhabdus* isolates inferred from gyrB gene. The numbers at the nodes represent bootstrap proportion value (1000 replicates, 50% or more). GenBank accession numbers and strain codes were given along with each species

and MBC values ranging from 7.81–3.90 mg/ml and 15.62–3.90 mg/ml, respectively. The highest activity of *Xenorhabdus* isolates was observed at the same value of MIC and MBC (3.90 mg/ml) with *X. vietnamensis* and *X. stockiae* against *E. coli* (ATCC 10536) and *K. pneumoniae* (ATCC 10031), respectively. In addition, both the *Xenorhabdus* isolates show the lowest activity against *B. subtilis* (ATCC 11774) with a MIC value of 7.81 mg/ml and an MBC value of 15. 62 mg/ml. Therefore, as per the observed MIC and MBC values, the overall activities of *Photorhabdus* extracts were

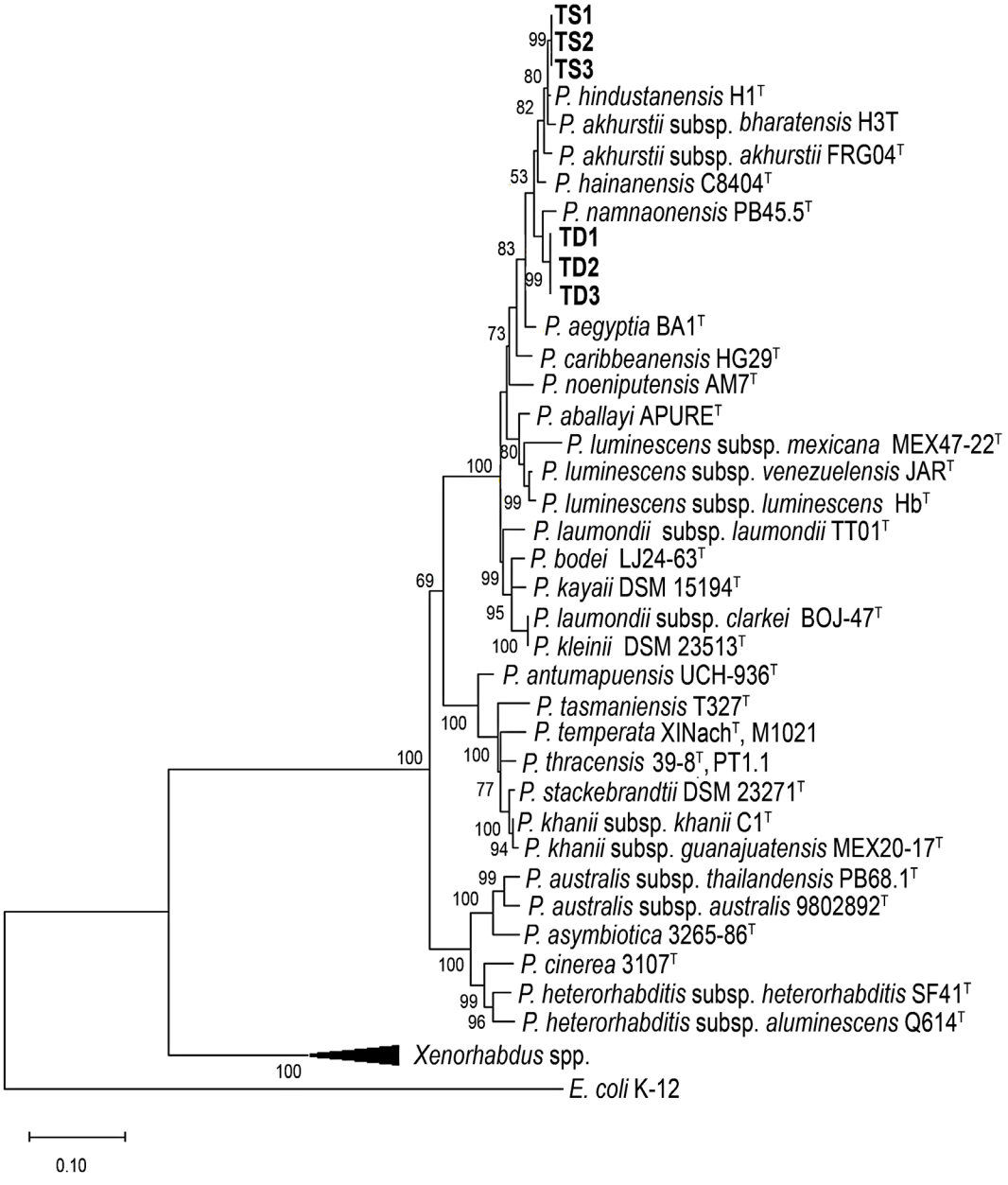

Figure 5. Maximum likelihood tree of *Photorhabdus* derived from two concatenated protein coding genes (recA and gyrB). The numbers at the nodes represent bootstrap proportion value (1000 replicates, 50% or more) and strain codes were given along with each species

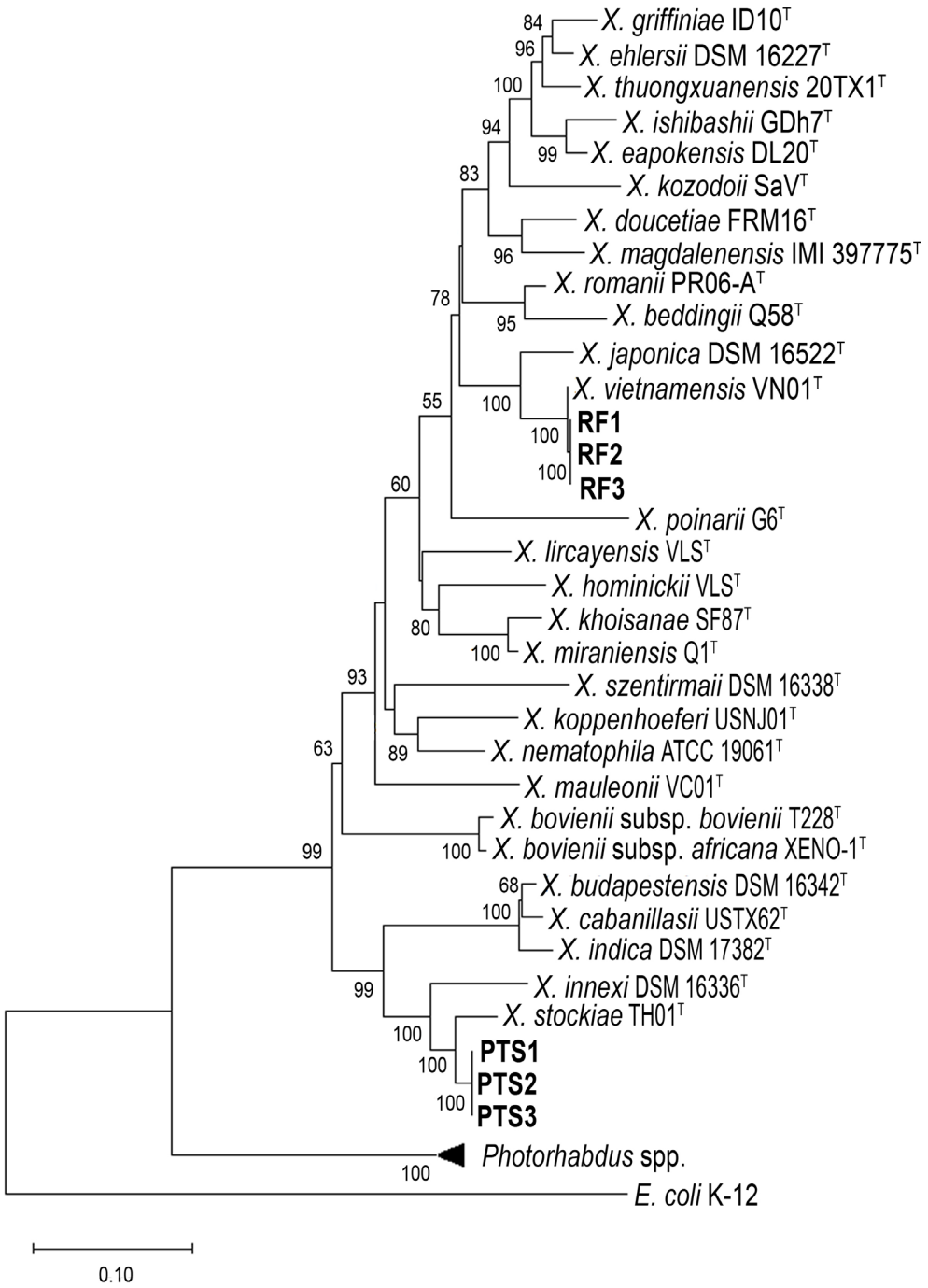

Figure 6. Maximum likelihood tree of *Xenorhabdus* derived from two concatenated protein coding genes (recA and gyrB). The numbers at the nodes represent bootstrap proportion value (1000 replicates, 50% or more) and strain codes were given along with each species

higher against the test pathogenic organisms as compared to *Xenorhabdus* isolates.

DISCUSSION

The subsequent analysis of the ML tree inferred from recA and gyrB revealed that the two *Xenorhabdus*, RF and PTS isolates, clustered cohesively with the previously identified *X. vietnamensis* and *X. stockiae* respectively, and thus belong to it. In contrast, some complications occurred to resolve the exact identity of the two *Photorhabdus* isolates (TS and TD). Lalramchuani et al.,[32] though forming a deep split between the two isolates, identified both the isolates as *P. luminescens* subsp. *akhurstii* using 16S rRNA gene. However, further analysis and reconstruction of the phylogenetic tree using the two housekeeping genes (recA and gyrB) revealed that TD isolates belong to *P. namnaonensis*. Analysis of the two genes further revealed that a consistent gap still exists between the two isolates by splitting into

Table. 3 MIC and MBC of bacterial extracts against pathogenic bacteria (mg ml^{-1})

Bacterial strain	*E. coli* (ATCC 10536)		*K. pneumoniae* (ATCC 10031)		*P. aeruginosa* (ATCC 10145)		*B. subtilis* (ATCC 11774)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
TS	3.90	3.90	1.95	3.90	1.95	1.95	3.90	7.81
TD	1.95	3.90	1.95	1.95	3.90	3.90	3.90	7.81
RF	3.90	3.90	3.90	7.81	3.90	7.81	7.81	15.62
PTS	7.81	7.81	3.90	3.90	3.90	7.81	7.81	15.62

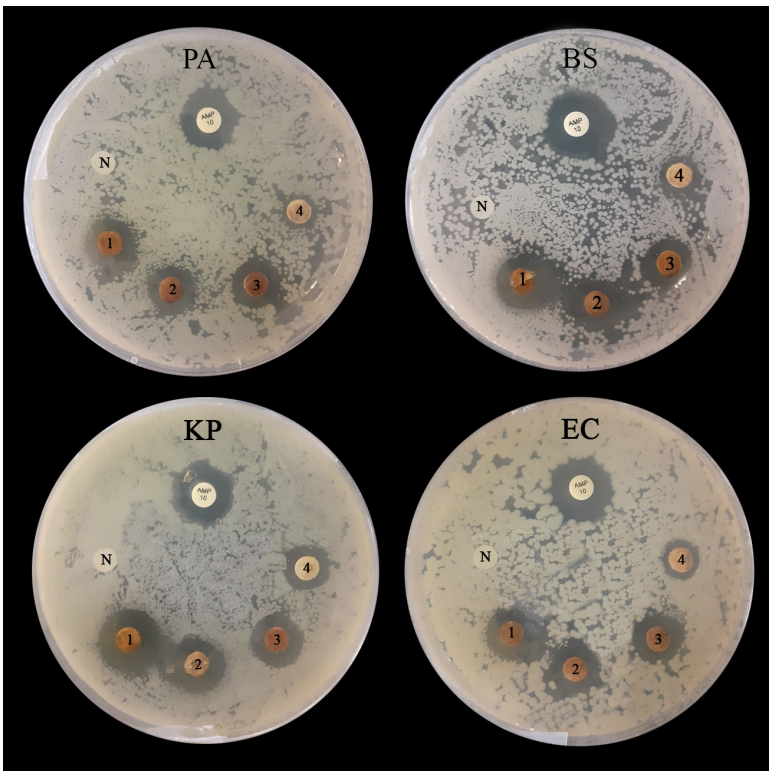

Figure 7. Antibacterial activities of bacterial isolates against pathogenic bacteria using disk diffusion method. (PA) *P. aeruginosa* (ATCC 10145, (BS) *Bacillus subtilis* (ATCC 11774), (KP) *K. pneumoniae* (ATCC 10031, (EC) *E. coli* (ATCC 10536), (1) TS, (2) TD, (3) RF, (4) PTS, (AMP) Ampicillin standard disk (10 µg) and (N) Negative control (DMSO)

two sub-clades. The *Photorhabdus* (TS) isolate undoubtedly belongs to *P. hindustanensis* as it clustered cohesively with the type species of *P. hindustanensis* H1^T (PUWT01). Meanwhile, the *Photorhabdus* (TD) isolate showed a deep split from *Photorhabdus* (TS) isolate clade and clustered closely with *P. namnaonensis* PB45.5^T (LOIC01) when analyzed with recA gene. However, analysis using another coding gene, gyrB revealed that TD isolates clustered closely with *P. hainanensis* (RCWD01). Therefore, the closer relationship of TD isolates with *P. hainanensis* (RCWD01) when analyzed with gyrB gene leads to confusion while there is a considerable separation between the two using another gene. The occurrence of this type of discrepancy might be the result of evolutionary pressure within different genes.[30] It is thus clear that phylogenetic analysis using a single gene causes incongruence regarding species identification, particularly in closely related species. Moreover, the concatenated ML tree indicated that the TD isolates and *P. namnaonensis* PB45.5^T clustered together cohesively, thereby suggesting them to be similar species.

Variations of activities including the degree of inhibition against the tested pathogenic organisms were observed among the isolates. This may be attributed to several factors such as the production of secondary active substances by *Photorhabdus* and *Xenorhabdus*, including media used for culture, medium pH, temperature, inoculation volume, fermentation time, rotary speed[33] and bacterium–nematode affected insect cadaver conditions.[34] These conditions may result in variability in the activity outcomes of bacterial extracts among various workers worldwide.

Consistent with our study, the more efficient antibacterial activities of *Photorhabdus*, compared to *Xenorhabdus* isolates, against several disease-causing bacteria i.e., *E. coli*, *P. aeruginosa*, and *K. pneumoniae* were also reported in previous studies.[19-21] The compound, lumicin from *P. luminescens* subsp. *akhurstii* showed satisfying activity against *E. coli*.[35] The high potency of *Photorhabdus* isolates against targeted microbes might be due to the production of several bioactive compounds including isopropylstilbenes and ethylstilbenes derivatives.[14, 15] Furthermore, *Photorhabdus* spp. are also known to produce broad-spectrum antibiotic properties including carbapenem, which is a prominent class of β-lactam antibiotics, responsible for activity against *E. coli*, *K. pneumoniae*, and *E. cloacae*.[36]

Xenorhabdus spp. are recognized for producing a wide range of chemicals having considerable antibacterial, antifungal, insecticidal and nematicidal activities.[33] *X. stockiae* extracellular metabolites show bactericidal activity against mastitis associated pathogens such as *E. coli* and *B. subtilis and S. aureus*.[37,38] and further could inhibit the growth of *P. aeruginosa,* but failed to inhibit *E. coli* and *K. pneumoniae.*[20] We have observed that the extract of *X. stockiae* suppressed the growth of all the tested pathogenic strains, including *E. coli* and *K. pneumoniae,* contradicting Muangpat et al.[20] However, the inhibition is comparatively lower compared to other studied isolates. Moreover, comparing the two *Xenorhabdus* isolates, the extract of *X. vietnamensis* showed higher activity than *X. stockiae* against all the tested pathogenic microorganisms. The potency of the genus *Xenorhabdus* against several microbes is due to the presence of compounds; fabclavine,[39] dithiolopyrrolone derivative[40], indole derivative compounds,[41] xenocin,[42] etc. The present report on the potential antibiotic activity possessed by *X. vietnamensis* adds another important data on antibiotic activity existing among the genus *Xenorhabdus*.

CONCLUSION

The current study focuses on the occurrence and identification of symbiotic bacteria in Mizoram, North-east India, which is part of an Indo-Burma biodiversity hotspot region. This study provides information on the symbiont's ability to function against specific harmful microorganisms. Among the isolates, we observed that *Photorhabdus* isolates were found to be more active compared with *Xenorhabdus* isolates. However, detailed analysis and studies need to be carried on to increase the knowledge of the exact genetic information, the composition of chemical compounds, and the mode of action against other microbes since these extracted metabolites could be a promising antibiotic in the future. This finding will pave the way for the identification

and investigation of certain symbiotic bacteria including their metabolites for the treatment of various diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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