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Isolation, Identification and Antimicrobial Activities of Four Bacteria Associated with Entomopathogenic Nematodes, *Steinernema* spp.

Othman M. Alzahrani (iD)

Department of Biology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia.

Abstract

Recently, the search for novel products derived from natural sources has become necessary due to the decreasing effectiveness of current antibiotics in treating bacteria that are antibiotic-resistant. In this context, it is well known that entomopathogenic bacteria (EPBs) produce a broad range of secondary metabolites with antibacterial activities. Therefore, an in-vitro trial was conducted to isolate and identify non-symbiotic bacteria associated with entomopathogenic nematodes, *Steinernema* spp. and evaluate the antibacterial activity against four antibiotic-resistant bacteria. Four bacterial isolates, i.e. *Pseudomonas alcaligenes* ST-1, *Paenibacillus barcinonensis* ST-2, *Bacillus mojavensis* ST-3, and *Bacillus megaterium* ST-4 were non-symbiotically isolated from the heamolymph of dead *Steinernema*-infected *Galleria mellonella* larvae and molecularly characterized. The bacterial cells and filtrates from *P. alcaligenes* ST-1 strongly inhibited the growth of *Staphylococcus aureus* through disk diffusion (43 mm), minimum inhibitory concentration (2.5 µL/mL), and minimal bactericidal concentration (5 µL/mL) assay. Conclusively, the direct application of endogenous *Steinernema*-associated EPB as an antibacterial agent for antibiotic-resistant bacteria looked promising.

Keywords: Non-symbiotic Entomopathogenic Bacteria, Molecular Identification, Antibiotic-resistant Bacteria, Antibacterial Activity

*Correspondence: o.alzahrani@tu.edu.sa

INTRODUCTION

Antibiotic resistance is currently one of the most serious worldwide health challenges. The abuse of antibiotics in both medicine and agriculture in industrialized and developing countries frequently results in antibiotic resistance mechanisms, which threaten modern medicine by decreasing the potency of therapeutically active drugs.[1-3] Antibiotic-resistant bacteria including *Staphylococcus aureus, Enterobacter cloacae, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumannii, Enterococcus faecalis,* and *Bacillus cereus* have become an emerging public health problem.[4] Bacteria have been exposed to long-term selective pressure due to the overuse of antibiotics, which has led to antibiotic resistance, making the bacteria difficult to eradicate. As a result, the expense of healthcare increases, illnesses last longer, more expensive drugs are used, and preventive and treatment are more effective. To effectively treat infections, antibiotics are utilized as a broad antimicrobial therapy. There are currently fewer antibiotics available that can prevent drug resistance, putting us at risk of running out of effective antibiotics in the future.[2-5] Antibiotic-resistant bacteria, which are anticipated to cause roughly 10 million annual deaths by 2050, are difficult to quantify accurately.[6] Therefore, antibiotic-resistant bacteria require alternative therapies. One such alternate strategy involves biological molecules derived from bacterial or natural sources. Gram-negative bacteria belonging to the Enterobacteriaceae family, *Xenorhabdus* and *Photorhabdus* are symbiotically associated with the infected juveniles (IJs) of entomopathogenic nematodes (EPNs) belonging to the *Steinernema* and *Heterorhabditis genera,* respectively.[7] During IJ penetration, the non-symbiotic bacteria, *Pseudomonas, Stenotrophomonas, Alcaligenes, Achromobacter, Pseudochrobactrum, Ochrobactrum, Brevundimonas, Deftia,* etc. are spontaneously "hitchhiked" into IJ vectors through the cuticle or intercuticular space and injected into the insect hemocoel.[8] It has been shown that bacterial resources for the synthesis of antimicrobial substances are either symbiotic or non-symbiotic bacteria associated with EPNs.[8,9] Their cell and filtrate activities successfully inhibit the growth of *S. aureus* and S. pyogenes,[10,11] *Botrytis cinerea* and *Bacillus subtilis,*[12] *K. pneumoniae, E. coli* and *E. coloacae,*[13] *Fusicladium effusum,*[14] *Phytophthora capsici, Bacillus anthracis* and *Rhizoctonia solani.*[15] These bacteria generate a wide spectrum of secondary metabolites, some of which have cytotoxic, insecticidal, and antibacterial properties.[11,16-18] The following substances have been shown to be antimicrobial: 3,5-dihydroxy-4-isopropystilbene,[12] 1-carbapen- 2-em-3-carboxylic acid,[13] benzaldehyde,[15] 3-hydroxy-2-isopropyl-5-phenethylphenyl carbamate,[19] 2-isopropyl-5-(3-phenyl-2-oxiranyl0-benzene-1,3diol,[20] and chaiyaphumine.[21] However, there is limited information on the antibiotic activity of the bacteria associated with EPNs from southwestern Saudi Arabia. This study's goals were to isolate and identify non-symbiotic EPBs associated with *Steinernema* spp.; analyze their phylogenetic variability; and test the antibacterial potency of the identified strains against four antibi-otic-resistant bacteria: *S. aureus, E. cloacae, E. coli,* and *B. cereus* using the method of disk diffusion, minimum inhibitory concentration (MIC), and minimal bactericidal concentration (MBC).

MATERIALS AND METHODS

Isolation of *Steinernema*-associated bacteria

EPNs, *Steinernema* spp. were previously isolated and morphologically identified[22] by Prof. Dr. Ahmed Noureldeen, Biology Departement, Faculty of Sciences, Taif University, Taif, Saudi Arabia. Using the method described by Vitta et al.,[23] non-symbiotic bacteria were isolated from the haemolymph of dead Galleria mellonella larvae that had been infected with the IJs of *Steinernema* spp.[23] In Brief, the dead *G. mellonella* larvae were surface-sterilized by washing in 100% ethanol for 1 min, and then they were deposited in a sterile Petri dish to dry. After that, a sterile-sharp needle was used to cut the third segment from the larvae's head, allowing a flow of symbiotic or non-symbiotic bacteria-containing haemolymph to emerge. The haemolymph samples were distributed and streaked over NBTA media (nutrient agar with 0.004% triphenyl tetrazolium chloride and 0.025% bromothymol blue) using a sterile loop, and then incubated at 28°C for 48 h.[24] Regularly, bacteria were cultured every

24 h until they produced pure, isolated colonies, after which they were refrigerated for subsequent work at -80°C with 20% glycerol (v/v). A single colony of each bacterial isolate was seeded in 5 mL of Luria-Bertani (LB) broth and cultured with agitation at 220 rpm overnight at 28°C to create the cell-free conditioned filtrates or cell suspensions. Furthermore, 100-mL aliquots of culture were shaken at room temperature for 24 h before being placed to flasks containing 400 mL of the same media and shaken for five days at 200 rpm. The supernatant and bacterial pellets were obtained after centrifuging the multiplied bacterial culture (13,000 rpm for 30 min) at 4°C. In order to get a cell-free filtrate, the supernatant was filtered using a 0.22 μm Millipore filter, and the pellet was then resuspended in sterile distilled water. In addition to obtain concentrations of 100, 80, 60, 40, 20, 10, 5, and 2.5 μL/mL, the filtrate was further diluted with sterile distilled water and kept at 4°C. Using a spectrophotometer, the bacterial cell suspension was adjusted at OD_{600} to 1.0. The bacterial suspension had a concentration of $1×10^7$ (CFU/mL) after using a 10-fold serial dilution spread plate.

Identification of *Steinernema*-associated bacteria DNA extraction from bacterial isolates

Using the Bacterial Genomic DNA Miniprep Kit, the genomic DNA of the isolated bacteria was obtained from the bacterial pellets (QIAprep Spin Miniprep Kit). Prior to use in a PCR, the bacterial genomic DNA was kept in a fridge at -20°C.

PCR Amplification of 16S rRNA Gene

In the PCR reactions, universal oligonucleotide primers were used: 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), and 1492R (5' TACGGT TACCTTGTTACGACTT-3').[25] The final reaction volume was 50 μL and contained 1 μL of bacterial DNA, 22 μL of ddH_2O, and 1 μL of each primer. In accordance with the manufacturer instruction, this mixture was added to the GoTaq® Green Master Mix (Promega, Madison, WI, USA). Then, carry out the following procedure using a C1000 TouchTM Thermal Cycler (Bio-Rad, Munich, Germany): A thermocycler was employed to complete 36 cycles, each consisting of three steps: denaturation (95°C for 1 min), annealing (54°C for 1 min), extension (72°C for 3 min), and final extension (72°C for 7 min).[26] Conventional electrophoresis was used to analyze amplified products and identify bands. By using universal primer, the amplified products' sizes were 1465 bp which indicates that the band is the 16S rRNA gene.

Sequencing of 16S rRNA gene

For four isolates that were packed in accordance with the National Health Research Ethics Committee's approval and in accordance with the sequencing company's guidelines, DNA purification and Standard Sanger sequencing were carried out (Macrogen Inc. Seoul, Korea). Under accession numbers (OP860651 to OP860654) on NCBI's website (https://www.ncbi.nlm.nih.gov), the following sequences have been added.

Bioinformatic analysis

The Finch TV program was used to visualize nucleotide sequence isolates (version 1.4.0). The web program nucleotide BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was used to explore Genbank databases for nucleotide sequence similarity.[27] The BioEdit program (version 7.2) was used to investigate closely related sequences from NCBI and subject them to multiple sequence alignment.[28] MEGA software was utilized to create the Neighbour-Joining phylogenetic tree with default settings (http://www.megasoftware.net/index.html).[29]

Antibiotic-resistant bacteria preparation

For antibacterial activity test, four isolates of antibiotic-resistant bacteria, *Staphylococcus aureus, Enterobacter cloacae, Escherichia coli,* and *Bacillus cereus* were employed. The Mueller-Hinton agar (MHA) was streaked with these bacteria, and it was then incubated at 37°C for 24 h. The turbidity was adjusted to 0.5 McFarland standards after dissolving a single colony in 0.85% sodium chloride (NaCl). For the disk diffusion test, a sample of the bacterial suspension in the amount of 100 μL was spread on MHA.[30]

Antibacterial activity

To initially evaluate the antibacterial activity of the four non-symbiotic bacterial cell suspensions or filtrates against antibiotic-resistant

bacteria, 20 μL of cell suspension (10^7 CFU/mL) or cell-free supernatant (200 μL/mL) from each isolate was dropped on a Mueller-Hinton (MH) agar plate containing bacteria resistant to antibiotics. After that, the plates were incubated for 24 h at 37°C. Positive results were read in a clean zone from the margin of a non-symbiotic bacterial growing colony. For the disc sensitivity test, EPB isolates that potentially inhibited at least one antibiotic-resistant bacterium were chosen.

Disk diffusion method

Ten microliters of each filtrate concentration at 200 μL/mL or cell suspension concentration at 10^7 CFU/mL from the four bacterial isolates were poured into sterile 6 mm paper discs, which were subsequently placed on the MHA plated with antibiotic-resistant isolates. Ampicillin-containing antibiotic disc was served as a positive control, while distilled water-containing disc was employed as a negative control. After that, the plates were incubated for 24 h at 37°C. A ruler was used to measure the clear zone's diameter (millimeter), which reflects the zone of inhibition.

Minimum Inhibitory Concentrations (MIC) and Minimal Bactericidal Concentrations (MBC)

Bacterial filtrates (Cell-free conditioned media) with the best disk diffusion results were subsequently assessed using the broth microdilution method to determine minimum inhibitory concentrations (MIC). In a 96-well micro titer plate, eight serial dilutions of the bacterial filtrate (100, 80, 60, 40, 20, 10, 5, and 2.5 μL/mL) were carried out. As a control, antibiotic-resistant bacteria were maintained in sterile Mueller-Hinton (MH) broth. For 24 h., plates were then incubated at 37°C. The MIC is the lowest concentration of filtrate at which the bacteria do not appear to be growing in the well. The minimal bactericidal

concentrations (MBC) were also assessed. 96-well MIC micro titer plates with ten microliters from each well were sub-cultured onto MHA plates. The plates were then incubated for 24 h at 37°C. Each filtrate's lowest concentration at which bacteria did not grow was classified as MBC. MIC and MBC assay were tested thrice.

Statistical analysis

The results were presented as mean ± standard error (M ± SE). Using the COSTAT program, the data were statistically evaluated by one-way analysis of variance (ANOVA), followed by multiple comparison, and significance was determined by P-values less than 0.05.

RESULTS

Phylogenetic analysis of 16S rRNA gene Sequences

The accession numbers of the four isolated bacteria non-symbiotically associated with *Steinernema* spp. were OP860651– OP860654 as appeared in Table 1. The number of base pairs in the 16S rDNA sequences of the bacterial isolates studied varied significantly and identified as *Pseudomonas alcaligenes, Paenibacillus barcinonensis, Bacillus mojavensis* and *Bacillus megaterium*. The different bacterial isolates showed homology in the nucleotide sequence from 98 to 100%. To elucidate the genetic relatedness between the bacterial isolates and the related sequence of strains obtained from the NCBI database on the basis of the sequencing analysis of the 16S rDNA gene, a phylogenetic tree was constructed. The phylogeny tree based on the obtained 16S rDNA sequences was divided into two main clusters (Figure 1). The first cluster was divided into two subclusters. The first one contained *Bacillus megaterium* ST-4 which was closed similar to *Bacillus megaterium* MT182961

Table 1. The NCBI BLAST query for bacterial isolates

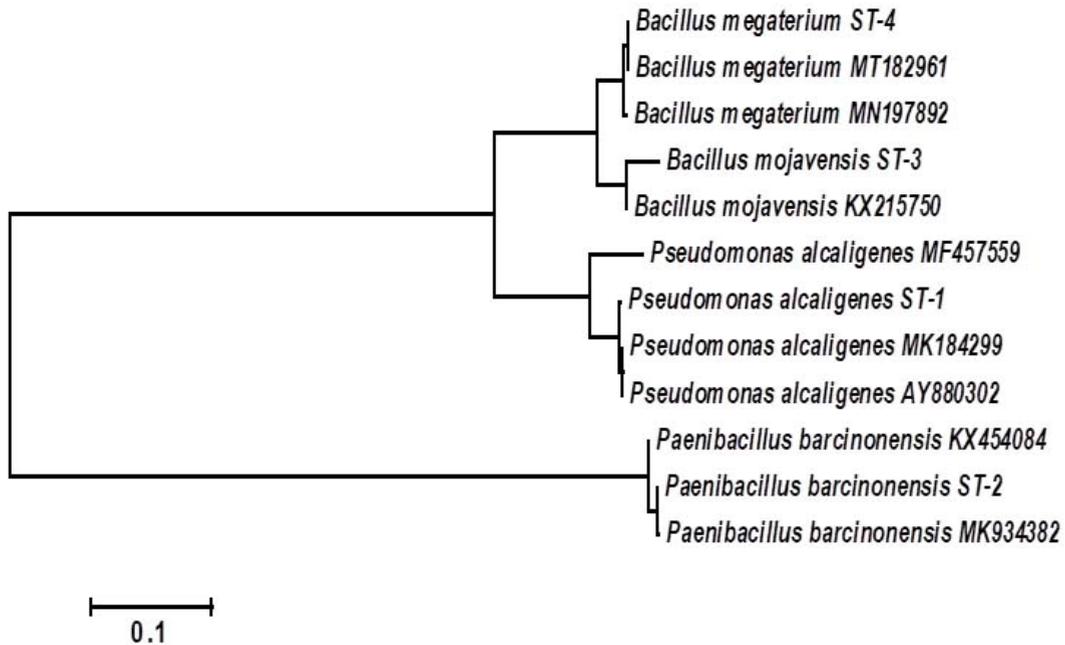| Isolates	Species	Query coverage %	E value	Ident %	Accession number
ST-1	*Pseudomonas alcaligenes*	100.00	0.00	99.00	OP860651
ST-2	*Paenibacillus barcinonensis*	100.00	0.00	100.00	OP860652
ST-3	*Bacillus mojavensis*	99.00	0.00	98.00	OP860653
ST-4	*Bacillus megaterium*	100.00	0.00	99.00	OP860654

Figure 1. Neighbour-Joining phylogeny based on 16S rRNA gene sequences of non-symbiotic bacterial isolates related to *Steinernema* spp.

and *Bacillus megaterium* MN197892 with a similarity ratio of 100 and 98 %, respectively. On the same subcluster *Bacillus mojavensis* ST-3 was found closed similarity to *Bacillus mojavensis* KX215750 with about a similarity ratio of 98%. Moreover, the second subcluster contained *Pseudomonas alcaligenes* ST-1 which was closed similar to *Pseudomonas alcaligenes* MK184299 and *Pseudomonas alcaligenes* MF457559 with a similarity ratio of 99 and 98 %, respectively. On the other hand, the second main cluster was contained *Paenibacillus barcinonensis* ST-2 which was closed similar to *Paenibacillus barcinonensis* MK934382 and *Paenibacillus barcinonensis* KX454084 with a similarity ratio of 99 and 98 %, respectively.

Bioactivity
Screening of EPB isolates against antibiotic-resistant bacteria

The data presented in Table 2 show that the whole cell culture and the filtrates of Steinerne-ma-associated bacteria had inhibition effect on tested antibiotic-resistant bacteria which varied from weak to strong inhibition. The antibiotic-resistant bacteria *S. aureus, E. cloacae,* and *E. coli* were susceptible to three of the EPB

isolates, whereas, *B. cereus* was sensitive to two of the bacterial isolates. Among all tested bacterial isolates, the strongest inhibition activities have been recorded by *P. alcaligenes* ST-1 and *B. megaterium* ST-4 cells and filtrates against *S. aureus*, while only cells of those bacteria have the same effects on *E. coli*. However, cells and filtrates of *P. alcaligenes* ST-1 reported moderate inhibition on *E. cloacae* growth, whereas, *B. megaterium* ST-4 cells and filtrates gave weak inhibition on the same bacterium growth. No inhibition activities have been noticed when *E. coli* and *B. cereus* bacteria exposed to *P. barcinonensis* ST-2 cells or filtrates, while all of the antibiotic-resistant bacteria except *E. coli* had resistance to *B. mojavensis* ST-3 (Table 2).

Antibacterial activity using disk diffusion method

The antibacterial activities of either cells or filtrates of the four isolated non-symbiotic bacteria associated with *Steinernema* spp. were confirmed by disk diffusion method (Table 3, Figure 2). *P. alcaligenes* ST-1, *P. barcinonensis* ST-2, *B. mojavensis* ST-3, and *B. megaterium* ST-4 bacterial cells at 10^7 CFU/mL and cell free supernatant at 200 µL/mL caused inhibition zone

(means ± SE) ranging from 14.4 to 43.0 mm, 0.0 to 16.6 mm, 0.0 to 10.5 mm, and 9.8 to 38.1 mm, respectively (P < 0.05). Bacterial cells of the four isolated strains were more effective against all of examined antibiotic-resistant bacteria than bacterial filtrates, inducing a mean clear zone of 12.96 mm for the cells and 10.21 mm for the filtrates (P < 0.05). The largest inhibition zone (43.0 mm) was significantly recorded when *S. aureus* bacteria were exposed to *P. alcaligenes* ST-1 cells, while it inhibited the growth of *E. cloacae*, *E. coli*, and *B. cereus* with the clear zone averaged 24.0, 31.3, and 22.8 mm, respectively. However, the cell free supernatant of *P. alcaligenes* ST-1 could inhibit the growth of *S. aureus, E. cloacae, E. coli,* and *B. cereus* with the clear zone 36.1, 20.5, 26.6, and 14.4 mm, respectively. Furthermore, the

cells and filtrates of *B. megaterium* ST-4 were the second most toxic to the examined bacteria with the inhibition zone of 38.1 and 32.5 mm, 13.1 and 9.8 mm, 30.3 and 25.0 mm, and 20.1 and 13.5 mm for *S. aureus*, *E. cloacae, E. coli, and B. cereus,* respectively. In contrast, the smallest inhibition zone was recorded from *E. coli* (6.4 mm) treated with filtrates of *B. mojavensis* ST-3. Moreover, no inhibition activity was recorded from *B. mojavensis* ST-3 against *S. aureus*, *E. cloacae,* and *B. cereus,* and *P. barcinonensis* ST-2 against *E. coli,* and *B. cereus* (Table 3).

Minimum Inhibitory Concentrations (MIC)

The MIC values of the four isolated non-symbiotic bacteria associated with EPN *Steinernema* spp. were also determined against

Table 2. Antibacterial activity of four bacterial species cells or filtrates on antibiotic-resistant bacteria

Bacterial species	Bacterial suspension type	Inhibit the growth of antibiotic-resistant bacteria[a]			
		S. aureus	*E. cloacae*	*E. coli*	*B. cereus*
Pseudomonas alcaligenes ST-1	Cells	+++	++	+++	++
	Filtrates	+++	++	++	+
Paenibacillus barcinonensis ST-2	Cells	++	+	-	-
	Filtrates	+	+	-	-
Bacillus mojavensis ST-3	Cells	-	-	+	-
	Filtrates	-	-	+	-
Bacillus megaterium ST-4	Cells	+++	+	+++	++
	Filtrates	+++	+	++	+

[a]No inhibition (-), weak inhibition (+), moderate inhibition (++), strong inhibition (+++)

Table 3. Antibacterial activity of four bacterial species cells or filtrates on antibiotic-resistant bacteria as assessed by disk diffusion

Bacterial species	Bacterial suspension type	Inhibition Zone (mm)[a]			
		S. aureus	*E. cloacae*	*E. coli*	*B. cereus*
Pseudomonas alcaligenes ST-1	Cells	43.0±2.07	24.0±1.51	31.3±1.67	22.8±1.67
	Filtrates	36.1±2.42	20.5±1.77	26.6±1.41	14.4±0.74
Paenibacillus barcinonensis ST-2	Cells	16.6±0.74	9.3±1.03	0.0±0.0	0.0±0.0
	Filtrates	13.1±1.25	6.3±1.03	0.0±0.0	0.0±0.0
Bacillus mojavensis ST-3	Cells	0.0±0.0	0.0±0.0	10.5±1.2	0.0±0.0
	Filtrates	0.0±0.0	0.0±0.0	6.4±1.41	0.0±0.0
Bacillus megaterium ST-4	Cells	38.1±1.49	13.1±1.13	30.3±1.04	20.1±1.25
	Filtrates	32.5±1.41	9.8±1.04	25.0±1.20	13.5±0.93
Ampicillin		0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

[a]Four repetitions, each with two plates, were used in this experiment to represent each treatment. Numbers in each column indicated to inhibition zone diameter ± standard error
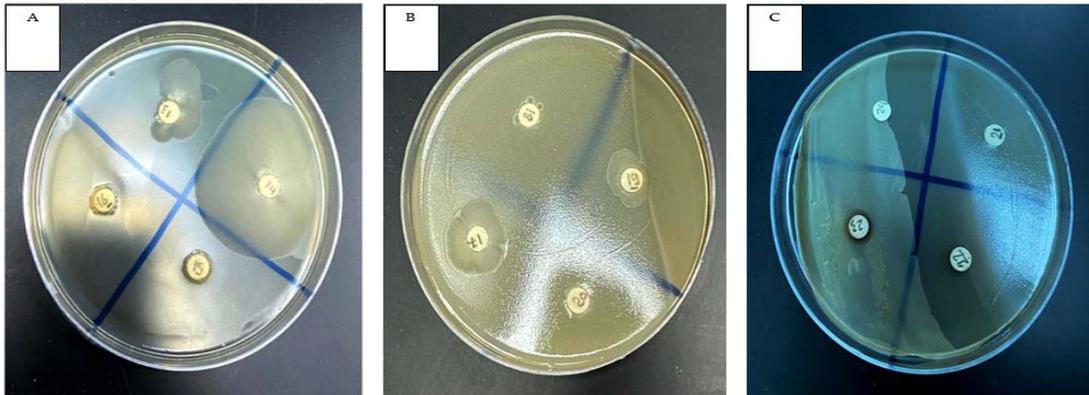
Figure 2. Disk diffusion method of bacterial cell-free supernatant of non-symbiotic EPBs against antibiotic-resistant bacterium *S. aureus.* Clear zone of *S. aureus* after exposure to bacterial filtrate from *P. alcaligenes* ST-1 (A14), *B. megaterium* ST-4 (B17), *P. barcinonensis* ST-2 (B19), *B. mojavensis* ST-3 (B20), antibiotic disc (C21) and negative control (C22)

four bacterial strains as indicated in Table 4. EPB filtrates with MICs ranging from 2.5 to 80 μL/mL inhibited the growth of antibiotic-resistant bacteria. The microorganism that recorded highest sensitivity towards *P. alcaligenes* ST-1 filtrate was *S. aureus* (2.5 μL/mL), followed by *E. coli* (5 μL/mL), then *E. cloacae* (20 μL/mL), and *B. cereus* (40 μL/mL). All of the testes bacteria were susceptible to *B. megaterium* ST-4 filtrate, which had the strongest effects (10 μL/mL) against *S. aureus* and *E. coli.*, followed by *E. cloacae* (40 μL/mL), and *B. cereus* (60 μL/mL), respectively. *P. barcinonensis* ST-2 presented activity against *S. aureus* (60 μL/mL) and *E. cloacae* (80 μL/mL), however, *B. mojavensis* ST-3 bacterial filtrate was active only against *E. coli* with the MIC value (80 μL/mL).

Minimal Bactericidal Concentrations (MBC)

Likewise, it seemed that the effective MIC also corresponds to the bacteria's effective bactericidal concentration (Table 5). The antibiotic-resistant bacterium *S. aureus* was the most susceptible to all filtrates except *B. mojavensis* ST-3 with MBCs ranging from 5 to 60 μL/mL. The activity of *P. alcaligenes* ST-1 filtrate against *S. aureus* was lower than that of all other tested bacteria with the MBC (5 μL/mL), followed by *E. coli* (10 μL/mL), while it recorded 40 and 60 μL/mL for *E. cloacae*, and *B. cereus*, respectively. E. cloaca was the least sensitive bacteria suppressing by *P. barcinonensis* ST-2 and scored highest MBC value (100 μL/mL).

DISCUSSION

This study's early stage was successful in recovering and isolating non-symbiotic EPB from *Steinernema* spp. Four *Steinernema*-associated bacteria were identified. These results are in line with those found by,[9] who isolated five EPBs associated with *Steinernema feltiae* and evaluated

Table 4. Minimum inhibitory concentration of bacterial filtrates (μL/mL) against antibiotic-resistant bacteria

Bacterial species	MIC (μL/mL)[a]			
	S. aureus	*E. cloacae*	*E. coli*	*B. cereus*
Pseudomonas alcaligenes ST-1	2.5	20	5	40
Paenibacillus barcinonensis ST-2	60	80	ND	ND
Bacillus mojavensis ST-3	ND	ND	80	ND
Bacillus megaterium ST-4	10	40	10	60

[a]Each treatment in this experiment had three repetitions. Numbers in each column indicated to minimum inhibitory concentration of each non-symbiotic bacterium. ND= Not detected

Table 5. Minimal bactericidal concentrations of EPB filtrates (μL/mL) against antibiotic-resistant bacteria

Bacterial species	MBC (μL/mL)[a]			
	S. aureus	*E. cloacae*	*E. coli*	*B. cereus*
Pseudomonas alcaligenes ST-1	5	40	10	60
Paenibacillus barcinonensis ST-2	60	100	ND	ND
Bacillus mojavensis ST-3	ND	ND	80	ND
Bacillus megaterium ST-4	10	60	20	80

[a]Each treatment in this experiment was represented by three replicates. Numbers in each column indicated to minimal bactericidal concentration of each non-symbiotic bacterium. ND= Not detected

their potentials against two aphid insects, *Aphis punicae* and *Aphis illinoisensis*. Accordingly, my investigations confirmed the previous results of,[31] who isolated in the same region *Photorhabdus* sp. and *Xenorhabdus* sp. from the *Heterorhabditis* sp. and *Steinernema* sp., respectively, and documented their complex activities against the root-knot nematode, *Meloidogyne incognita*, which infects pomegranate under greenhouse conditions. The present study's findings definitely demonstrated that bacterial culture and 16S rDNA sequence analysis offer a novel method for comparing different bacterial community patterns. Based on a phylogenetic tree analysis, the four isolated species of *Steinernema*-associated bacteria discovered here were named separately and molecularly identified as *P. alcaligenes* ST-1, *P. barcinonensis* ST-2, *B. mojavensis* ST-3, and *B. megaterium* ST-4. These findings agreed with those of other research.[32-34,25] Data had a high bootstrap percentage and demonstrated how the most isolated strains related to one another. The other isolates, in contrast, had longer branch lengths, which were in line with earlier discoveries, and this was explained by the incomplete 16S rDNA sequences.[34-36] In the present study, the four isolated bacteria from the heamolymph of insect *G. mellonella* were non-symbiotically related to *Steinernema* spp. infection, and have been first identified in Saudi Arabia. These outcomes are consistent with those that[8] have previously recorded, on four species of *Steinernema* and their associated bacteria. Regarding the antibacterial activity, it was evident that the four bacterial isolates cells were stronger than bacterial filtrates in their ability to combat antibiotic-resistant bacteria, which showed low

toxicity. The different isolates of EPBs have been reported with variation in antibacterial activity. This might be as a result of each bacterium's ability to synthesize therapeutic metabolites or the sensitivity of bacteria that are resistant to antibiotics. Among the examined bacterial isolates, *P. alcaligenes* ST-1 and *B. megaterium* ST-4 cells and filtrates exhibited the strongest inhibitory effect, whereas, *B. mojavensis* ST-3 displayed the weakest inhibition activity. These results are in consent with those of[37] who noted that high diversity of antimicrobial components present in *B. megaterium* strains make them prospective sources of novel antibiotics produced by bacilli and demonstrate efficacy against test strains that are resistant to pharmaceutical antibiotics (gram-negative bacteria P. aeruginosa ATCC 27853 and *E. coli* ATCC 25922, glycopeptide-resistant leuconostoc, and methicillin-resistant staphylococcus). These findings are also in agreement with those of[38,39] who mentioned that b-Sitosterol, behenic acid, phenylacetic acid, and other identified *Bacillus megaterium* L2 compounds show promise as potential natural antimicrobial agents by destroying cell membrane integrity, damaging cell structures, affecting cell metabolism, and inhibiting protein synthesis to exhibit an antibacterial effect. Similarly, a novel cyclic peptide compound from *B. megaterium* was quantified and showed broad spectrum antimicrobial activity against both gram positive and gram negative bacteria, with minimum inhibitory concentrations of 6.25, 3.125, 1.0, 0.5, and 0.25 μg/mL against *S. aureus* ATCC25923, *P. aeruginosa* ATCC27853, *E. coli* ATCC35218, *Salmonella typhi* ATCC19430, and *Micrococcus luteus* ATCC10240, respectively, with no activity

against *C. albicans* ATCC10231.[40] The obtained data also revealed that *S. aureus* and *E. cloacae* were sensitive to all isolated bacterial cells and filtrates except *B. mojavensis* ST-3; however, *E. coli* was susceptible to all isolates except *P. barcinonensis* ST-2; meanwhile, *B. cereus* showed sensitivity to *P. alcaligenes* ST-1 and *B. megaterium* ST-4 only. Accordingly, based on mean inhibition zone values, the toxicity caused by studied EPBs against tested antibiotic-resistant bacteria could be categorized in the descending order: *P. alcaligenes* ST-1 > *B. megaterium* ST-4 > *P. barcinonensis* ST-2 > *B. mojavensis* ST-3; whereas, the sensitivity of the antibiotic-resistant bacteria could be arranged as *S. aureus* > *E. coli* > *E. cloacae* > *B. cereus*. These results are in the same context with the previous studies regarding the EPBs, *Photorahbdus* and *Xenorhabdus* those showed that *P. luminescens* could inhibit the growth of drug-resistant and clinical isolates *B. subtilis*, *E. coli*, and *S. aureus* RN4220.[13] Additionally, *S. aureus* ATCC20475, *S. aureus* strain PB36, and *S. aureus* strain PB57 were also vulnerable to inhibitory activity of *Photorhabdus* extracts.[41] Likewise, *Xenorhabdus* synthesized xenocoumacin[42] and amicoumacin[43] derivatives, both of which were discovered to be effective antibiotics against *S. aureus*.[10] However, isopropylstilbene,[44,45] which all *Photorhabdus* spp. produced exhibited a wide range of biological properties including antibiotic action against *S. aureus* and *E. coli*.[19] The results showed that the EPBs *P. barcinonensis* ST-2 and *B. mojavensis* ST-3 have less bactericidal potential against antibiotic-resistant bacteria based on the MIC and MBC. In contrast, earlier investigations show that *B. mojavensis*, and its secreted compounds, lipopeptides, and fengycin effectively suppress a number of bacterial and fungal strains.[46,47] Similarly, Singh et al.[48] screened the inhibitory activity of *Paenibacilllus* sp. and found that *S. aureus* (ATCC 25923), *E. coli* (MTCC 1610), *C. albicans* (MTCC 224), and *Bacillus subtilis* (ATCC 6633), were all inhibited by the filtrate and/or crude fermentation extract of fifteen isolates, suggesting the antibacterial activity due to compound bacitracin A. Also, peptides isolated and purified from *Paenibacillus peoriae* IBSD35 cell-free supernatant showed a high and broad spectrum of antimicrobial activity.[49] Here, the author reported that the non-symbionts

EPBs were isolated for the first time from *Steinernema*-infected *G. mellonella* heamolymph and considered as antibacterial agents. Of these, the symbiont, *Xenorhabdus* was not recorded in current study. This might be as a result of the bacterium's limited distribution. This is consistent with the findings of earlier research in Taif region, Saudi Arabia which recorded the nematicidal and insecticidal[9,31,22] activities. Overall, the obtained results revealed that different species of antibiotic-resistant bacteria were affected differently by EPB isolates. This might be caused by each bacterium's ability to generate valuable metabolites or by antibiotic-resistant bacteria's sensitivity to the specific metabolites manufactured by each non-symbiont.

CONCLUSION

In the current study, four bacterial isolates were non-symbiotically associated with *Steinernema* spp., and be identified as the first recorded at Taif, Saudi Arabia. Their antibacterial efficacy was evaluated against four important antibiotic-resistant bacteria. *P. alcaligenes* ST-1 amongst acts as an excellent antibacterial agent to the four tested antibiotic-resistant bacteria. The findings provide a reliable base for the discovery of a novel bioactive compound.

DATA AVAILABILITY

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

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by the author.

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