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Biochemical, Molecular and Antibacterial Agent Characterization for an Egyptian Isolate of Genus *Xenorhabdus*

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ABSTRACT

Pure isolated culture of *Steinernema carpocapsae* entomopathogenic nematodes which were obtained using single egg-mass technique were used. This isolate was processed through last-instar larvae of the greater wax moth, *Galleria mellonella* several times to obtain pure cultures. One isolate of *Xenorhabdus* sp. bacteria was isolated from the haemolymph of last infected instar *G. mellonella* larvae. Bacterial colonies were established on nutrient agar by streaking haemolymph from this insect. During isolation bacteria were partially identified by colony characters on Brain heart agar, NBTA and MacConkey agar media. Bacterial cells were grown in Brain heart infusion broth for liquid cultures and on Brain heart infusion agar for solid cultures. Bacteria were identified depending on morphological, biochemical and physiological characteristics. Bacteria were identification by 16SrRNAgene partial sequencing, the results of sequence alignment and phylogenetic tree drawing revealed that the isolated strain was *X. nematophila* as it gave 100 % similarity with the corresponding bacterial type worldwide. The antibacterial activity was always detained in the protein fraction of the bacterial culture supernatant. The antimicrobial protein was isolated and tested for their antimicrobial activity against some pathogenic bacteria, *Salmonella typhemurium* was the most sensitive Gram negative bacteria, while *Staphylococcus aureus* was the most sensitive Gram positive tested bacteria. The antibacterial activity was detected with protein fractions weighting about 200 KDa.

KEWWORDS: *Xenorhabdus*, Antibacterial proteins, Entomopathogenic nematodes, 16s rRNA gene, Pathogenic bacteria

INTRODUCTION

Symbionts of bacteria belonging to entomopathogenic nematodes of the families Heterorhabditidae and Steinernematidae are members of the family Enterobacteriaceae and belong to the genera *Photorhabdus* and *Xenorhabdus*, respectively **(Thomas and Poinar, 1979)**.

Xenorhabdus sp. is a Gram-negative symbiotic bacteria of an entomopathogenic nematode *Steinernema carpocapsae* and is belonging to family Enterobacteraceae **(Poinar, 1966; Wang et al., 2008)**. After entering the nematodes through their natural openings they deliver the symbiotic bacteria from their intestine to the hemocoel of the insect hosts **(Kaya and Gaugler, 1993)**. The bacteria will be logarithmically replicates in the insect haemolymph, then cause a depression to the host immunity against both the symbiotic nematodes and the bacteria themselves by inhibiting phospholipase A of the insect host **(Park et al., 2003)**. During such condition the *Xenorhabdus* sp. will produce an antibiotic like substances known as narrow spectral bacteriocins **(Boemare et al., 1992)**. The monoxenic state leads to lethal septicemia of the target insect, which is required for development of the symbiotic nematodes in the cadaver **(Forst et al., 1997)**.

A study carried out by **Wolf et al. (2008)** suggests that the antibiotic compounds produced by *Xenorhabdus* to preserve the insect cadaver from others bacteria may be used in the aim of controlling mastitis caused by bacteria. Indeed, *X. budapestensis*, *X. szentirmaii* and *X. nematofila* appear to be efficient against pathogens such as *Staphylocuccus aureus* and *Escherichia coli*. The bacteria can produce antibiosis compounds in vivo and in vitro. *X. nematophilus* also can inhibit the growth of *Phytophthora infestants*, an Oomycota that causes potato blight **(Yang et al., 2001)**.

There is a DNA region of *X. nematophilus* found to encode for insecticidal protein, chitinase, and other pathogenic attributes. This suggests that the toxins of *X. nematophilus* are made up of multiple

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components like polypeptides. When the secretions of *X. nematophilus* are centrifuged, the particulate fraction of the insecticidal proteins are more active than the soluble fraction. This has implication in use of this microbe in biocontrol as it will dictate the conditions needed for it to be most effective **(Khandelwal and Banerjee-Bhatnagar, 2003)**.

The 16s rRNA gene has been revealed as a good marker to determine diversity among bacteria and currently all species description should now include the 16s rRNA sequence species. However, 16S rRNA evolves so slowly and may not always be high enough to distinguish closely related strains **(Adams et al., 2006)**. **Agazadeh et al. (2010)** constructed phylogenetic trees based on the sequences of 16S rRNAgene which showed that all of the studied *Xenorhabdus* sp. were identified as *X. bovienii*, *X. nematophilus* and *X. budapestensis*.

Therefore, the aim of this investigation is to isolate and characterize a strain of *Xenorhabdus* species and study the nature of its antimicrobial agent.

MATERIALS AND METHODS

Nematodes used

Steinernema carpocapsae nematode symbiotic for the bacteria *Xenorhabdus* sp., was kindly provided from the Department of Parasitology and Animal Diseases, National Research Centre, Dokki, Egypt. The nematodes were maintaining in vitro by passaging through the larvae last-instars of *Galleria mellonella* according to **Dutky (1964)**.

Bacteria isolation

Bacteria were isolated from the haemolymph of last instar *G. mellonella* larvae infected with *Steinernema carpocapsae* nematode isolate according to **Akhurst (1982)**. Bacterial colonies were established on nutrient agar by streaking haemolymph from the infected insects. During isolation the colonies were examined on Brain heart agar, NBTA (2.3% nutrient agar, 0.0025% bromothymol blue, 0.004% 2,3,5-triphenyltetrazolium) or MacConkey agar media. Bacterial cells were grown in Brain heart infusion broth for liquid cultures and on Brain heart infusion agar for solid cultures.

Bacteria identification

Bacterial identification was carried out depending on morphological, colony, biochemical and physiological characteristics **(Sneath, 1986)**.

Isolated bacteria were identified by 16S rRNA gene as described by**Jung et al. (2006)**. Total genomic DNA was extracted from the bacterial isolate with the aid of Bacterial Genomic Miniprep Kit (Sigma-Aldrich Chemie GmbH, Germany) using the method described in product manual. Eluted DNA was used directly for the Polymerase chain reaction (PCR) as stated by **Weisburg et al. (1991)**.

Two universal primers for *Xenorhabdus* sp. **(Weisburg et al., 1991)** were used for PCR amplification of the 16S rRNA partial gene sequence of the bacterial isolate. Forward and reverse primers were '5-AGG GYT ACC TTA CGA CTT-3' and '5-GTT TGA TCC TGG CTC AG-3', respectively. The PCR reaction mixture was added to each tube as follows: 5µl of 10x PCR buffer, 0.8µl of dNTP mix (12.5 mM each), 2µl of 25 pmol forward primer, 2µl of 25 pmol reverse primer, 0.25µl of Taq DNA polymerase (5 units/µl), 2µlMgCl 25 mM solution, 2 µl of template DNA and 34.95µlof sterile distilled water. Conditions were, preheating at 95°C for 2 min, 30 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min and final extension was carried out at 72°C for 5 min.

For PCR product analysis, 1.5% agarose gel was used and electrophoresis was carried out in Sub-Cell DNA apparatus (Bio-Rad® Lab., USA) at 80 V. The amplified gene band was visualized on an UV Transilluminator and photographed by Gel Documentation System (AlphaImager® TM1220, Documentation and Analysis system, Canada).

Sequencing and Phylogenetic analysis

Sequencing was carried out at Gene Analysis Unit (VACSERA, Agouza, Cairo, Egypt) using ABI Prism® BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). Sequencing products were resolved in an automated sequencer model 310 (Applied Biosystems, USA).

DNA partial sequences of 16SrDNA from different worldwide *Xenorhabdus* sp. isolates and *Escherichia coli* control sequence were collected from GeneBank **(http://www.ncbi.nlm.nih.gov)** as demonstrated in **Table (1)**. These sequences were compared with the nucleotide sequence of the understudy

bacterial isolate to draw homology tree. Molecular analysis and tree drawing were carried out using the DNAMAN Software, version 8.0 (Lynnon Corp., Canada).

Table 1.Source of *Xenorhabdus* sp.16SrDNA sequences used for comparisons

Organism	Country of origin	Accession number	Strain
X. nematophila	USA	NR_119150	DSM 3370
X. hominickii	France	DQ205449	KR05
X. hominickii	South Korea	GU480985	Mt. Jiri
X. miraniensis	France	DQ211713	Q1
X. szentirmaii	Germany	NR_042328	DSM 16338
X. ehlersii	Germany	NR_042327	DSM 16337
X. bovienii	France	NR_074382	SS-2004
X. miraniensis	France	NR_043644	Q1
X. indica	Germany	NR_114965	28
X. hominickii	Japan	AB507815	KmYb11
X. khoisanae	USA	NR_117921	SF87
X. romanii	France	NR_043647	PR06-A
X. nematophila	Japan	NR_115575	ATCC 19061
X. nematophila	France	NR_102822	ATCC 19061
X. nematophila	Iran	FJ640983	4
Escherichia coli	USA	NR_024570	U 5/41

Antibacterial activity of different culture supernatant fractions

Cell free bacterial culture different fractions were obtained as described by **Abou El-Hag and El-Sadawy (2008)**. Bacteria was grown on brain heart infusion broth at 28°C for 48h. The cell free Supernatant was collected from liquid culture by centrifugation at 10,000 rpm for 30 min at 4°C.The supernatant was filtrated using 0.45 µmMillipore filter under aseptic conditions for the remaining bacterial removing. The fractions were separated according to their chemical nature by dissolving the lipid in equal volume of chloroform at room temperature for 60 min with contentious shaking and the lipid layer was obtained by aspiration with syringe. The protein content was separated by precipitation using ammonium sulfate as mentioned by **Carbral et al. (2004)**, ammonium sulfate was gradually added with stirring till 90% saturation. The precipitate was collected by centrifugation at 10,000 rpm and then dissolved in 50mM sodium phosphate buffer, pH (7.2). The ammonium sulfate precipitation was dialyzed against distilled water overnight. The remaining water soluble carbohydrates was concentrated by sucrose overnight followed by dialyses against distilled water overnight.

The antibacterial activities in lipid, protein and carbohydrate fractions were tested depending on sensitivity test as mentioned by **Cherif et al. (2003)**.In a clean sterile 9 cm Petri-dish 6 ml of sterile agar was poured. Ten microliter of tested sample were spotted on the agar and dried for 30 min at 37°C. Another 6 ml of agar contain 1 ml of 10^8bacterial suspension of tested pathogenic bacteria was mixed and poured at 40-45°C and incubated at 30 °C / 48 h. The clear zone diameters were measured around spots and Norfloxacine antibiotic disks were used as a control. The tested pathogens were: *Escherichia coli*, *Pseudomonas flourecence*, *Salmonella typhemurium*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterooccus fecalis* (kindly obtained from Dept. of Dermatology, Fac. of Medicine, Ain Shams Univ.).

Antibacterial activity of protein fractions

The protein fraction detected to posies the antibacterial activity was analyzed by 12 % Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE) to identify the active proteins and number of protein fractions as described by as described by **Laemmli (1970)**. The produced protein fraction was also passed through Vivaspin® 6 centrifugal concentrators (Vivaproducts, Inc., USA) with different molecular weight cut-off (10, 30, 50, 100 kDa). The antibacterial activity was detected for each cut-off produced as previously mentioned. Fraction with antibacterial activity were analyzed using 12 % SDS-PAGE.

RESULTS

Bacteria isolation and identification

Microscopic examination of the produced bacterial colonies proved that they are short rods Gram positive cells. The resulted colonies on Brain Heart agar were cream white colonies with translucent appearance and on MacConky agar the bacteria were capable to adsorb the neutral red stain, while the growth on NBTA were capable to adsorb bromothymole blue and triphenyltetrazolium stain giving dark

blue-green colonies in alkaline pH. Some studied characteristics of the isolated bacteria are summarized in **Table (2)** proving that it can be related to genus *Xenorhabdus*.

The partially identified bacterial isolate was confirmed using PCR with specific primers for 16S rRNA partial gene sequence. PCR product was analyzed on 1.5 % agarose gel producing a band of about 1.4 Kbp which is the expected size of the understudy gene part **(Figure 1)**.

The partial 16S rRNA gene sequence of the isolated bacteria was aligned to the homologous sequences of *Xenorhabdus* sp. from different countries worldwide. Results of sequence alignment and homology tree drawing revealed that the isolated strain was *X. nematophila* as it gave 100 % similarity with the corresponding bacterial type worldwide **(Figure 2)**.

Table 2. Differential characteristics of the isolated *Xenorhabdus* Strain

Characteristics & Biochemical tests		Isolated *Xenorhabdus* Strain
Morphology		Short rods
Spores		-
Gram staining		-
Capsule		-
Motility		Motile
Pigments on nutrient agar		Brown
Oxygen requirements		Aerobic
Growth temp. (ºC):	4	-
	30	+
	37	+
	41	+
	50	-
Growth pH:	4	+
	5.7	+
	6.8	+
	9.0	+
Growth in NaCl (%):	5	+
	7	+
	10	-
Gelatin hydrolysis		+
Starch hydrolysis		+
Glucose		+
Maltose		+
Manitol		+
Lactose		+
Sucrose		+
Citrate		+
VogesProskauers (VP)		+
Indol		-
Nitrate reduction		+
Catalase		-
Oxidase		+
Urease		-
Blood agar haemolysis		-
L-Cystien hydrolysis		-

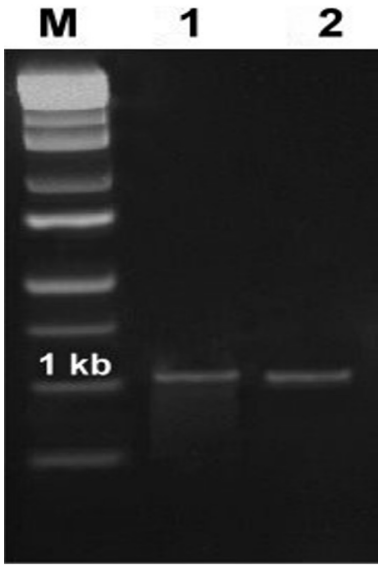

Figure 1. PCR product of *Xenorhabdus* sp. 16S rRNA partial gene sequence (1.4 kbp), Lane 1-2. Lane M: Lambda DNA/EcoRI+HindIII marker (Promega, USA).

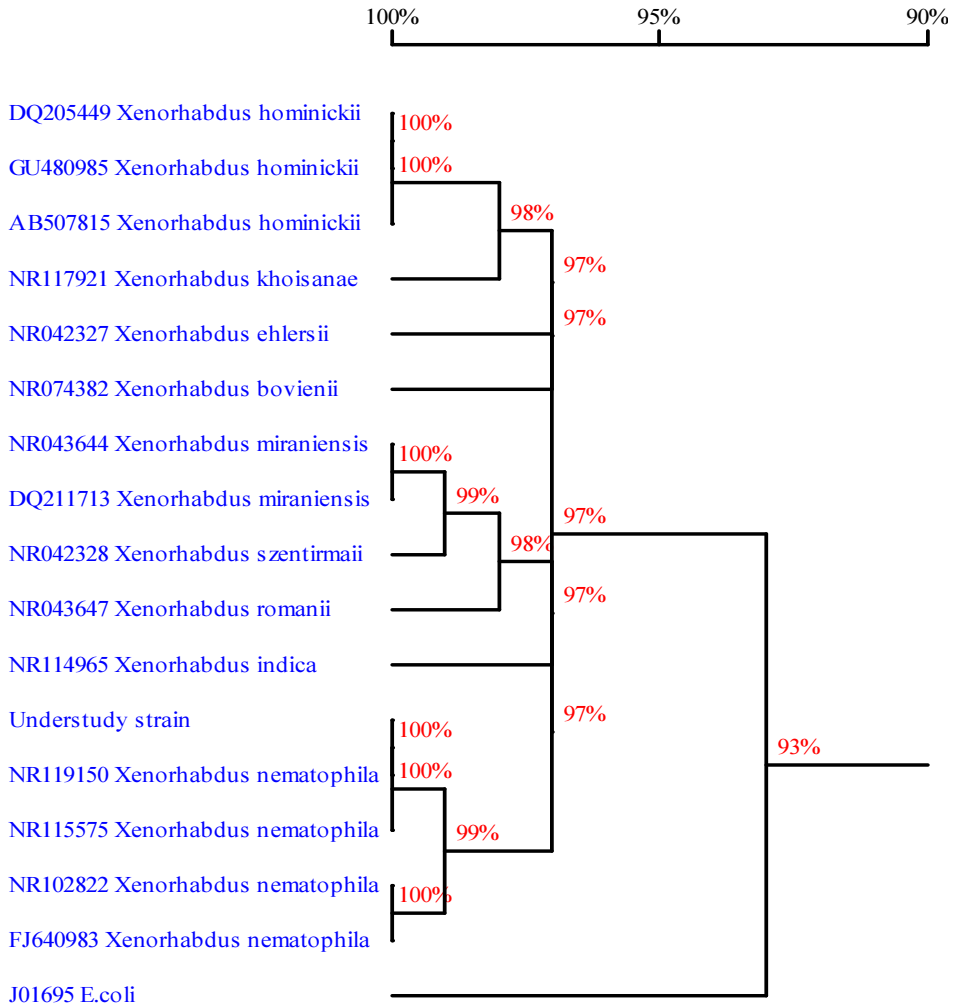

Figure 2. Homology tree and similarity degrees between the understudy *Xenorhabdus* isolate and some overseas isolates.

Antibacterial activity of different culture supernatant fractions

By testing the antibacterial activity of protein, lipid and carbohydrate fractions against Gram negative and positive pathogens it was clearly observed that the antibacterial activity was always detained in the protein fraction only. *Salmonella typhemurium* was the most sensitive Gram negative bacteria, while *Staphylococcus aureus* was the most sensitive Gram positive tested bacteria **(Table 3)**.

Table 3. Sensitivity test of the protein, lipid and carbohydrate *Xenorhabdus* sp. culture supernatant fractions against some pathogenic bacteria.

Tested pathogenic bacteria	Inhibition zones diameter (mm) with different *Xenorhabdus* sp. culture supernatant fractions			
	Protein	Carbohydrate	lipid	Norfloxacine (Control)
E. coli	10	-	-	20
P.flourecence	11	-	-	22
S.typhemurium	16	-	-	29
S. aureus	14	-	-	26
Str.pyogenes	9	-	-	18
Ent.fecalis	12	-	-	27

Antibacterial activity of protein fractions

The 12 % Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE) analysis of the total protein fraction extracted from *X. nematophila* supernatant cleared that it consist of about 22 protein bands **(Figure 3)**. However by using Vivaspine concentrators of different molecular weight cut-off (10, 30, 50, 100 kDa.) the antibacterial activity remained in the protein fraction of molecular weight ranging from 100 kDa. The 100kDaVivaspine concentrator maintained the active antibacterial protein fraction. Electrophoretic analysis of active 100kDaVivaspine concentrator product on 12% SDS-PAGE revealed that it consists of one protein band with molecular weight of about 200 kDa **(Figure 3)**.

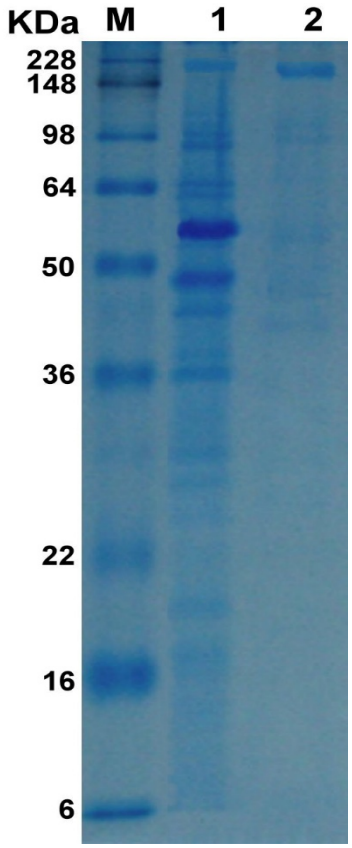

Figure 3. The SDS-PAGE analysis of the total protein fraction extracted from *X. nematophila* supernatant (Lane 1). Electrophoretic analysis of active 100 kDa Vivaspine concentrator product (about 200 KDa) (Lane 2). M: marker protein (Promega, USA).

DISCUSSION

Xenorhabdus nematophilus is a gram-negative bactera belonging to the family Enterobacteriaceae, and can be described as entomopathogenic. *X. nematophilus* is not found free living in the soil environment, but existing in a symbiotic relationship with insect-parasitizing nematodes of *Steinernema* sp. **(Xiangling et al., 2014)**.

In order to create an optimal growing environment for reproduction, the nematode needs the entomopathogenic bacterial symbiont. *X. nematophilus* will secrete proteinous compounds that kill the insect host, additional compounds include enzymes that bioconvert the insect host. Antimicrobial agents are also secreted that protect the insect carcass from other microbial infections. In the initial stages of infection of the insect host, *X. nematophilus* inhibits the growth of various fungal and bacterial parasites **(Ansari et al. 2005)**. The metabolites produced by the bacteria are found to have antifungal, nematicidal, or insecticidal effects. *X. nematophilus* limits competition so that the nematode and it can use the insect larvae carcass nutrients **(Xiangling et al., 2014, Khandelwal and Banerjee-Bhatnagar, 2003)**.

There is a DNA region of *X. nematophilus* found to encode for insecticidal protein, chitinase, and other pathogenic attributes. This suggests that the toxins of *X. nematophilus* are made up of multiple components (e.g. polypeptides) **(Floyd et al., 2002)**.

Depending on bacterial morphology, colony characters and some biochemical studies, the understudy bacteria were proved that it can be related to genus *Xenorhabdus*. The obtained results were in full harmony with data obtained before by **Abou El-Hag and El-Sadawy (2008)**.

Results of sequence alignment and homology tree drawing revealed that the isolated strain was *X. nematophila* as it gave 100 % similarity with the corresponding bacterial type worldwide. In harmony results were obtained by **Agazadeh et al. (2010)** who studied six strains of *Xenorhabdus* originating from several geographical isolates of entomopathogenic nematodes molecularly to distinguish their position among the well-defined worldwide species.

As found before by **Abou El-Hag and El-Sadawy (2008)**, and by testing the antibacterial activity of protein, lipid and carbohydrate fractions against Gram negative and positive pathogens it was clearly observed that the antibacterial activity was always detained in the protein fraction only. And by testing the analyzed proteins of the bacterial supernatant it was clearly found that a one band protein of about 200 KDa having the antibacterial effect.

Salmonella typhemurium was the most sensitive Gram negative bacteria, while *Staphylococcus aureus* was the most sensitive Gram positive tested bacteria. Results with same trend were also found by **Abou El-Hag and El-Sadawy (2008)**.

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