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MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *Xenorhabdus* nematophila BACTERIA SYMBIOTICALLY ASSOCIATED WITH THE ENTOMOPATHOGENIC NEMATODE STEINERNEMA CARPOCAPSAE

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ABSTRACT

The Xenorhabdus genus is newly defined as include a family of entomopathogenic nematodes and other big, rod-shaped, facultatively anaerobic bacteria. These bacteria are gram-negative. After worms introduce these bacteria, they often make their home in the intestines of nematodes or the insect host's body cavity. The bulk of the significant features connected with this family are possessed by the bacteria in this genus, which is why it is placed under the Enterobacteriaceae family. Notable features that set Xenorhabdus apart from other Enterobacteriaceae genera are its large cells, lack of nitrate conversion capability, tight association with endogenous nematodes, enteropathogenesis, and unique immunological characteristics. Poinar and Thomas's Xenorhabdus nematophilus comb.nov. is the officially recognized species. Isolated and studied using microbiological, biochemical, and molecular methods was the bacterial community linked to entomopathogenic nematode (EPN) infective juveniles (IJs), Steinernema sp. Based on their unique behavioral features, twelve bacterial isolates were collected from entomopathogenic nematode (EPN) infective juveniles (IJs).The 16sRNA analysis confirmed that the bacterial isolates were Xenorhabdus nematophila.Keywords: Symbiotic bacteria, entomopathogenic nematode, *Xenorhabdus nematophila*, Steinernema sp NBTA Media.

INTRODUCTION:

Nematodes are primarily free-living organisms that reside in aquatic environments or soil. Insects serve as hosts for several species of nematodes, and around 25% of all nematodes are parasitic to either plants or animals. Various types of relationships can exist, such as phoresy, symbiosis, commensalism, obligatory or facultative parasitism, and even parasitic worms that infest insects. These organisms can be often seen in nature, where they invade several areas of their hosts' body, including the exoskeleton, hemocoel, reproductive, digestive, or excretory systems. Among the numerous orders of nematodes linked with insects, the Heterophobia and the Steinernematidae are particularly fascinating (Filippov, 1934; Poinar, 1976). When discussing the practical aspects of hexapod management, it is more precise to refer to the nematodes in these two categories as entomopathogens, as they collaborate with symbiotic bacteria.

Common EPNs pose no harm to plants, humans, or higher animals. Due to their recognized insect selectivity and multicellular composition, EPNs are typically excluded from the registration screening process that is typically carried out on entomopathogenic organisms prior to their usage. Entomopathogenic nematodes (EPNs) are biocontrol agents that hold significant potential as an alternative to chemical pesticides.(Labaude and Griffin, 2018) because of their exceptional ability to identify, locate, infect, and cause the host insects (mostly lepidopteran and coleopteran species) to die quickly within a span of 48 hours.

A multitude of laboratories worldwide are actively utilizing this nematode-bacteria complex in the fields of agriculture, medicine, and industrial. Scientists specializing in ecology, evolution, biochemistry, molecular genetics, and symbiosis have identified nematode hosts and bacterial symbionts as a highly effective model system for their research (Burnell and Stock, 2000; Good-rich Blair and Clarke, 2007).

It is important to mention that the genus Xenorhabdus has a higher degree of similarity among its species compared to the genus Photorhabdus. However, the genus Xenorhabdus has a larger overall number of species. While there are 19 known species of Photorhabdus, Sajnaga and Kazimierczak have documented the existence of 26 distinct species of xenorhabdus. Possible explanation: The number of steinernema species, which are the reciprocal partner of xenorhabdus spp., is greater than the number of heterotrophic nematodes, which are the reciprocal spouse of photorhabdus spp. There are about 100 species of steinernema, but there are less than 20 species of heterotrophic nematodes.

Research has been conducted to isolate entomopathogenic nematodes (EPNs) from different agricultural settings, with a specific focus on insect pests. The references cited are Pervez et al., 2014 and Maru et al., 2007.Although there is abundant evidence of Xenorhabdus engaging with Steinernema spp. and Photorhabdus with Heterorhabditis spp., our understanding of the interactions between other related bacteria remains limited. Furthermore, apart from the typical symbionts, there are extra bacteria that either suppress or establish colonies in the corpses of nematodes and insects. Consequently, the present study focused on Steinernema sp., a native entomopathogenic nematode, with the objective of identifying and characterizing the symbiotic bacteria that coexist with it.

Every xenorhabdus strain has the ability to spontaneously develop two unique physiological states, phase I and phase II, in vitro.9) Various variants of the identical gene generate antibiotics, assimilate dyes on agar plates, and excrete a diverse range of proteins (including lipases and proteases). Phase II variations, however, lack or have dramatically reduced levels of these characteristics.Both forms are afflictions that impact the larvae of insects. However, phase I variants are specifically linked to infectious nematodes, which commonly infest insects. The precise function of the extracellular chemicals generated by xenorhabdus spp. in the symbiotic relationship and virulence of nematodes is not yet fully understood [3]. Nevertheless, it is hypothesized that these bacteria confer advantages to their host nematodes and participate in activities that are poisonous to insects [5,7].

Materials and methods:

ISOLATION OF BACTERIA (*XENORHABDUS NEMATOPHILUS*) FROM EPN (STEINERNEMA SP) (TCX)

Isolation of the bacteria associated with Steinernema sp. was accomplished through the application of Akhurst's method (1980). During the white trap procedure, the diseased cadaver of Corcyra cephalonica was utilized in order to successfully capture the recently obtained IJs. From the hemolymph of a Corcyra cephalonica that was infected with EPN, Xenorhabdus was isolated successfully. The hemolymph was plated on nutritional bromothymol “blue triphenyl tetrazolium chloride agar” (NBTA), and then it was allowed to incubate at a temperature of 25 degrees Celsius for a period of four days in complete darkness. When the most prevalent type of bacteria consumed bromothymol blue dye, the colours of the bacteria changed to blue. A single black-dark blue colony was used to inoculate each bacterium into an Erlenmeyer flask that was at least 100 milliliters in volume. The flasks were shaken for a full night at a

temperature of 25 degrees Celsius. The bacteria were transferred to 400 ml of the same medium the next day and agitated at a rate of 200 rotations per minute for five days to prepare the bacterial filtrate. The multiplying bacterial culture was centrifuged for 30 minutes at a speed of 13,000 revolutions per minute at a temperature of 4 degrees Celsius. To obtain a cell-free filtrate, pass the residue through a millipore filter with a pore dimension of 0.22 µm. The filtrate was held at four degrees Celsius for the duration of its needed period.

CHARACTERIZATION OF BACTERIAL ISOLATE (*XENORHABDUS NEMATOPHILUS*)

The pure cultures is analysed using biochemical and microbiological methods (Boemare and Akhurst,1988; Cappuccino and Scherman, 1999).

The bacteria exhibit unique colony formations on NBTA, a selective medium employed for preliminary identification, based on their colony morphology. Each isolate was streaked onto a sterile petriplate containing NBTA and cultivated for a duration of four days. The MacConkey agar medium was employed to differentiate Xenorhabdus from other microorganisms. The gram responses and cell shape of the bacterial isolates linked with EPNs were observed using a 100X microscope. The Gram staining procedure was performed on 24-hour-old cultures using the standard approach described by Peel et al. (1999).

Examining Biochemical Parameters: All of the isolates underwent the biochemical assays described by Albert et al. (1992) and Akhurst and Boemare (1983) using the following protocols: citrate utilization, urease, indole, methyl red, Vogues-proskauer, and Macconkey's agar.

The cultures utilized for the experiments were maintained at a temperature of 24ºC.We utilized peptone water shake cultures (consisting of 1% peptone and 0.5% NaCl) that had undergone a 24-hour incubation period in order to quantify cell quantities. Colony features were documented from 1-5 day old cultures of tergitol 7 agar with tri phenyl-tetrazolium chloride (T-7 + TTC agar), as well as from 24-hour old cultures of nutritional agar (NA).The bioluminescence was evaluated by subjecting NA cultures to a period of full darkness lasting 20 minutes. The motility broth for the 18-hour incubation period was made by combining 18.5 g of brain heart infusion and 5.0 g of glucose per liter of water. In Flagellum staining, the colonies cultivated on nutrient-rich media were exposed to a 10% solution of hydrogen peroxide to determine their catalase activity. Additionally, they were exposed to a 1% solution of tetramethyl-p-phenyldiamine dihydrochloride in water and 0.1% ascorbic acid to determine

their oxidase activity. The analysis focused on carbohydrates, specifically D-Arabinose and Esculin.

The bacterial symbiont showed no activity for the following enzymes: Enzymes such as "β-galactosidase", "arginine-dihydrolase", "lysine decarboxylase", "ornithine decarboxylase", "citrate assimilation", "H2S production", urease, "tryptophan deaminase", indole production, acetoin production" (as determined by the Voges-Proskauer test), gelatinase, "acid production from glucose, mannitol, inositol, sorbitol, "rhamnose", sucrose, "melibiose", "amygdalin", arabinose, and rham.

Molecular Characterization:

The alkaline lysis method was utilized in order to extract the genomic DNA from each of the twelve bacterial isolates that were associated with EPN. The primers (5'AAGGAGGGGATCCAGCCGCA3') and (5'GGAGAGTTAGATCTTGGCTCAG3') were acquired from Sigma-Aldrich in the United States and diluted in accordance with the protocols for the PCR tests. Primers were obtained from the "National Center for Biotechnology Information (NCBI)" database, which can be accessed online at http://www.ncbi.nlm.nih.gov. In order to carry out the "polymerase chain reaction" (PCR), fifty microliters of a 1X buffer, 1.5 "millimolar magnesium chloride" (MgCl2), and "two hundred microliters of deoxyribonucleotide triphosphates" (dNTPs) were utilized. In our experiment, we utilized a template DNA concentration of 50 ng/µl, 0.5 µM of both forward and reverse primers, and one unit of DNA polymerase from Genei Bangaluru. The 16S rRNA was denaturated at 95 degrees Celsius for five minutes as the first step prior to the amplification process. After that, there were 35 cycles of heating to 94 degrees Celsius for one minute, cooling to 55 degrees Celsius for thirty seconds, and then heating once more to 72 degrees Celsius for ten minutes. For the PCR control reactions, the same primers were utilized; however, the reactions were carried out without the presence of template DNA.A 0.8% TAE agarose gel was utilized in order to accomplish the separation of the 16SrRNA sequence from the PCR result. For the purpose of removing DNA fragments from agarose gel in a speedy and effective manner, the gel extraction kit manufactured by Eurofins was utilized. Through the utilization of nanodrop technology, Eurofins IT Solution India was able to sequence the eluted DNA and precisely estimate its concentration. Located in Bengaluru, India, Eurofins is a company. Altschul et al. (1990) state that the NCBI online tool was utilized in order to perform an analysis and evaluation of the acquired sequence in order to determine whether or not it exhibited homology.

Results and Discussion:

Morphological characterization of the isolated symbiotic bacteria:

The Xenorhabdus bacterial cells, as observed in Figure 1, exhibited a reduced size and were visibly stained purple, as indicated by the Gram staining technique. The studies conducted on the Xenorhabdus bacterium on NBTA (Nutrient Bromo thymol blue tetrazolium chloride agar media) revealed that the colony morphology of this bacterium is dark blue, convex, and unborate (Figure 2).

Figure 3 demonstrates that the Xenorhabdus colony cultivated on Eosine Methylene blue (EMB) media had a flat appearance and had a distinct green metallic luster. The genomic DNA of a bacteria was effectively isolated and amplified using 16SrRNA sequencing. The gene bank has stored the sequence under the accession number isOR633290 for Xenorhabdus nematophilus. The neighbor-joining method was employed to construct the phylogenetic tree, depicted in figure 4, using gene amplification as the basis.

Photorhabdus and Xenorhabdus are anaerobic, elongated, sporogenous, Gram-negative bacteria from the Enterobacteraceae family. Xenorhabdus is 0.3-2×2-10 µm in size, while Photorhabdus is 0.5×1-10 µm. Steinernema and Heterorhabditis are known for their symbiotic partnerships, in which both species benefit from the other's parasitic presence. Entomopathogenic bacteria thrive on artificial surfaces, where they generate and emit secondary metabolites (12-14). These chemicals interact with both Gram-positive and Gram-negative bacteria and have anticancer, anti-ulcer, antiviral, antifungal, and antibiotic activities.A symbiotic interaction exists between bacteria and nematodes, which allows the nematode to flourish. During Steinernema nematode infection, Xenorhabdus bacteria live in a particular sac just behind the basal bulb of the esophagus (15).When juveniles infected with Heterorhabditis examine their digestive systems, they will discover Photorhabdus bacteria (8).Despite the fact that they can reproduce independently, nematodes and bacteria work very closely together, with the bacterium exploiting its cellular barrier to kill just the insects it is especially targeting. The catalase test is commonly used to assess whether an organism is aerobic or anaerobic. We used biochemical assays such as urease, citrate utilization, methyl red, Mac Conkey, Veges-Proskauer, and the Indole Test—gold standard methods—to determine nterobacteriaceae family membership. Cowles and Goodrich-Blair (2008) anticipated the partnership based on the Steinernema species' sole bacterial symbiont, xenorhabdus nematophila. The current study used morphological, biochemical, and molecular

criteria to identify bacterial species. Numerous researchers have recently discovered new types of bacteria, including the well-known endosymbionts Photorabhdus sp. and Xenorhabdus sp. Torres-Barragan et al. (2011) and Shan et al. (2019) investigated four bacterial species related with EPN: Serratia marcescens, Achromobacter xylosoxidans, Providencia rettgeri, and Enterococcus mundtii. EPN's bacterial microbiota, also known as "frequently associated microbiota (FAM)," are pollutants detected as a result of human or animal handling or laboratory activity.However, when they began to appear often in the blood of impacted insects, things began to change. Researchers first identified these in 2014 (Singh et al).According to Pervez et al. (2020), Photorhabdus luminescens forms symbiotic partnerships with Heterorhabditis sp. (IISR-EPN09), Providentia vermicola, Pseudomonas entomophila, Alcaligenes aquatilis, and Acinetobacter faecalis.

Ogier et al. (2020) examined the microbiota of EPNs to study the relationship between symbiotic bacteria and Infective Juveniles (IJs) of Steinernema carpocapsae, S.feltiae, S.glaseri, and S.weiseri. They discovered many proteobacteria associated with these IJs.Ogier et al. (2023) stated that Xenorhabdus and Photorhabdus constitute the initial bacterial cycle of EPNs. The second bacterial circle, potentially consisting of more intricate bacterial communities, may participate in the parasite cycle by infiltrating the insect's body, transferring EPN (Entomopathogenic Nematodes), and proliferating in the soil. Previous studies have demonstrated that EPNs exhibit greater pathogenicity when combined with particular bacteria.In order to determine if anything is capable of causing disease, it is important to evaluate the ability of symbiotic organisms and the microorganisms they interact with to kill insects.

Conclusion:

Xenorhabdus nematophilus is the taxonomic classification of the Xenorhabdus strain obtained from Steinernema Carpocapsae.However, the manner in which these two groupings of organisms are characterized by their species varies from one other. Moreover, the formation of bacterial species may need a significantly greater degree of evolutionary alteration at the DNA level compared to the process of generating nematode species.Despite the fact that the bacterial symbionts of the nematodes Xenorhabdus and Steinernema belong to the same species at present, the co-speciation phenomenon has endured.

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Table 1

Comparison of xenorhabdus with other genera of the family Enterobacteriaceae

Genus	G+C Content of DNA(mol%)	H_2S Production	Citrate production	Motility	Cell size of rods (µm)	Colony colour	Association with EPN	Pathogenic for insects
xenorhabdus	43-44	-	-	+	0.8-2.0	Blue to blue green	+	+
Escherichia	50-51	-	-	±	1.1-1.5	Yellow or red	-	-
Salmonella	50-53	+	+	+	NG	Red	-	-
shigella	50-53	-	-	-	NG	Red	-	-
Enterobacter	52-59	-	+	+	NG	Red	-	-
Klebsiella	52-56	-	+	-	0.3-1.5	Red	-	-
Proteus	38-42	±	+	+	0.4-0.6	Red	-	-

Information regarding genera other than xenorhabdus was extracted from BERGEYS MANUAL (1) whenever it was accessible.

Data on genera other than “xenorhabdus” were collected using “The Difco Manual and Difco supplement”.

Table2

The biochemical and morphological traits of Xenorhabdus species:

SL.NO	CHARACTERISTICS	**BACTERIAL STRAIN (***Xenorhabdus nematophila)*
1.	Gram reaction	-
2.	Cell size (µm)	**5.2×2.3**
3.	Motility	+
4.	Peritrichous flagella	+
5.	Pigmentation	_
6.	Bioluminescence	_
7.	BTB binding	+
8.	Antibiotics	+
9.	Crystal Protein	+
10.	Swarming	+
11.	Lipase	+
12.	Gelatinase	+
13.	DNase	+
14.	Catalase	_
15.	Oxidase	_
16.	Urease	_
17.	Lecithinase	+
18	Citrate utilization test	
19.	Methyl red test	--
20.	Mac Conkey Agar test	**Pink**
21	Voges-Proskauer test	--
22.	Indole test	--

TCX -Tirunelveli District (Cashew Tree)-*Xenorhabdus nematophila*

+-Positive - -Negative

Table 3

SL.NO	Carbohydrates	Bacterial strains (TCX)
1.	D-Arabinose	-
2.	Esculin	-
3.	D-Fructose	+
4.	D-Galactose	-
5.	D-Glucose	+
6.	Inositol	+
7.	Insulin	-
8.	D-Lactose	-
9.	D-Maltose	+
10.	D-Mannitol	-
11.	D-Mannose	+
12.	D-Raffinose	-
13.	D-Sorbitol	-
14.	L-Sorbose	-
15.	D-Xylose	-

Acid production was defined by the pH Level in the test tube pH < 6.0 (+); pH 6.0-6.5 (+/-) and pH > 6.5 (-)

Table 4

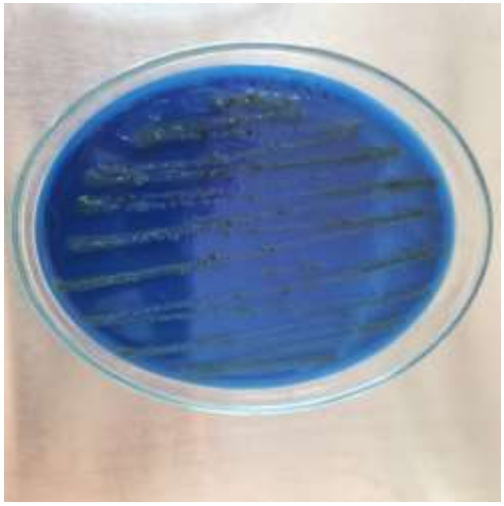
Utilization of carbon sources by Xenorhabdus (TCX) Strains

Sl.NO	Carbon source	Bacterial strains (TCX)
1.	Asparagine	+
2.	Cysteine	-
3.	Glycine	-
4.	Tyrosine	+
5.	Nicotinic acid	-
6.	Ethanol	-
7.	Methanol	-
8.	Inositol	+
9.	Mannose	+

10	D-Galactose	-
11.	D-Glucose	+
12.	D-Lactose	-
13.	D-Mannitol	-
14.	D-Sorbitol	-
15.	D-Ribose	+

Utilization of carbon (+) turbidity with in 6 days; (+/-) Delayed turbidity with in 6 days; (-) no turbidity over 6 days.

Rice moth larva cadavar



Xenorhabdus culture

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