

Isolation and Molecular Characterization of Symbiotic Bacteria *Xenorhabdus* Associated with Entomopathogenic Nematodes in Ghaziabad and Meerut, India

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Abstract: A survey for Entomopathogenic nematodes was conducted during 2013-2015. 100 soil samples collected from 20 different locations in Ghaziabad and Meerut, India. Eleven Entomopathogenic nematodes were isolated by insect bait method using fifth instar larvae of *Galleria mellonella* and 11 symbiotic bacteria were isolated from these Entomopathogenic nematodes. These were identified as three *Xenorhabdus stokiae* and eight *Xenorhabdus indica* based on molecular data.

Keyword: Symbiotic bacteria, *Xenorhabdus stokiae*, *Xenorhabdus indica*, NBTA, Phylogenetic

1. Introduction

The Entomopathogenic nematode (EPN) of genus *Steirnermema* (Travassos, 1927) is the most intensively studied group of nematodes associated with insects. They are obligate and lethal parasites of many insect-pests making them ideal candidates for use as biological control agent against crop pest (Gaugler 2002). These nematodes are symbiotically associated with bacteria from the family Enterobacteriaceae that are pathogenic to insect. Steinernematids are associated with *Xenorhabdus species* (Thomas and Poiner, 1979). The third stage infective juvenile is the only free-living stage that persists in the soil in search of a susceptible arthropod host. IJs makes their entries through the natural opening (mouth, anus, spiracles) and release the symbiotic bacteria into the insect hemocoel. The mutualistic bacterium propagates and produces substances that rapidly kill the host within 24-48h. and protect the cadaver from colonization by other microorganism. The nematode initiates its development, feeding on the bacterial cells and host tissues that have been metabolized by the bacterium and has 1-3 generations, depending on host size. When food resources become depleted, new IJs either remain at the site where they were produced or disperse in search of new hosts.

Xenorhabdus bacterial genera produce phenotypic variant cell types called primary form and secondary form. The primary form is the cell type naturally associated with the nematodes whereas the secondary form can arise spontaneously when the bacterial cultures are in the stationary non-growth stage and the secondary form revert to the primary form. The primary form produces antibiotics, adsorbs certain dyes and develops large intracellular inclusion composed of crystal proteins. Secondary form only weakly produces antibiotics, does not adsorb dyes 321, and produces intracellular inclusions inefficiently. The primary form is superior to the secondary form.

2. Material and Methods

Isolation and identification of symbiotic bacteria

Isolation of Entomopathogenic nematodes (EPNs) three *Steirnermema saimkayai* and eight *Steirnermema thermophilum* were isolated from Ghaziabad and Meerut district of western U.P, India using *G.mellonella* by baiting methods. Eleven native EPNs were transferred to the surface of a filter paper in 35-mm petri dishes and put the *G. mellonella* larvae in the dish. The dishes were incubated at 28°C for 48 h. The larvae cadavers were removed, rinsed in distilled water. In the sterile condition surface sterilized with 70% ethanol, flame and left to drying in a laminar.

Cadaver dissected using sterilized needles and scissors then a drop of hemolymph streaked on NBTA agar media plates (peptone- 1.25gm, beef extract- 0.75gm, Agar- 3.75gm, BTB- 0.0062gm, TTC- 0.01gm, NaCl- 1gm, Ph- 7.2, water- 250ml). NBTA agar media plates were sealed using parafilm and incubated at 28°C in the dark for 24h. Single colonies of bacteria were removed using a sterile loop and transferred to new NBTA agar plates and they were subcultured continually until the single colony appears. Each single colony was selected and inoculated to a Luria broth, the flask was then placed in a shaking incubator adjusted at 200 rpm for overnight at 28°C.

DNA extraction, PCR and phylogenetic analysis

Overnight 1ml of bacterial culture was transferred to 1.5 eppendorf tube. Total genomic DNA was extracted using a DNA isolation kit (Invitrogen Genomic DNA mini kit). Genomic DNA gene amplification was done by a PCR gradient thermocycler using primers. The total volume of one reaction PCR mixture was 25µl, containing 2.5µl of 10x buffer, 1.75 µl of 50mM MgCl₂, 1.25 µl of 10mM dNTPs, 1.25 µl of 100 p mole forward primer, 1.25 µl of 100 p mole reverse primer, 0.4 µl of 5 unit Taq enzyme, 16 µl of nuclease water and 0.5 µl of genomic DNA. The PCR condition were denaturation at 95°C for 3 min., followed by

35 cycles of 95°C for 45 sec., 58°C for 45 sec., 72°C for 2min. and final extension at 72°C for 10 min. The DNA band was excised and extracted from the gel by using QIAGEN (min elute) Gel extraction kit. The obtained sequence of *Xenorhabdus stokiae* and *Xenorhabdus indica* were compared with sequence of *Xenorhabdus stokiae* and *Xenorhabdus indica* species available in GenBank (NCBI). The DNA Sequences were edited using BioEdit (Hall,1999) with sequences of related species and new isolates. The evolutionary relationship of the isolated with 10 species/strains for *Xenorhabdus stokiae* and 10 species/strains for *Xenorhabdus indica* were evaluated (Swofford, 2002). Phylogenetic (Neighbor joining) analysis the sequence data were done using MEGA (Tamura et.al. 2007).

3. Results

Bacteria were recovered from all 11 samples. Suitable quantity of purified total DNA template was extracted from the overnight bacteria culture and DNA template was yielded from each extraction. The genomic DNA from the 11 samples using the (F-5GAGTAATGTCTGGAAACTGCC 3', R-5'

GTTAGCCGGTGCTGCTTCTG 3') primer were identified . The purified PCR-products were sequenced. The sequences from PCR-product were successfully obtained from all samples. Three sample in the BLAST search indicate similarity between *Xenorhabdus stokiae* and eight samples indicated similarities between *Xenorhabdus indica*. The evolutionary relationship of the isolates and other closely related species were evaluated. The phylogenetic analysis of the genomic DNA sequence data placed *Xenorhabdus stokiae* and *Xenorhabdus indica* isolates in a clade with other isolates of *Xenorhabdus stokiae* and *Xenorhabdus indica* species/strain.

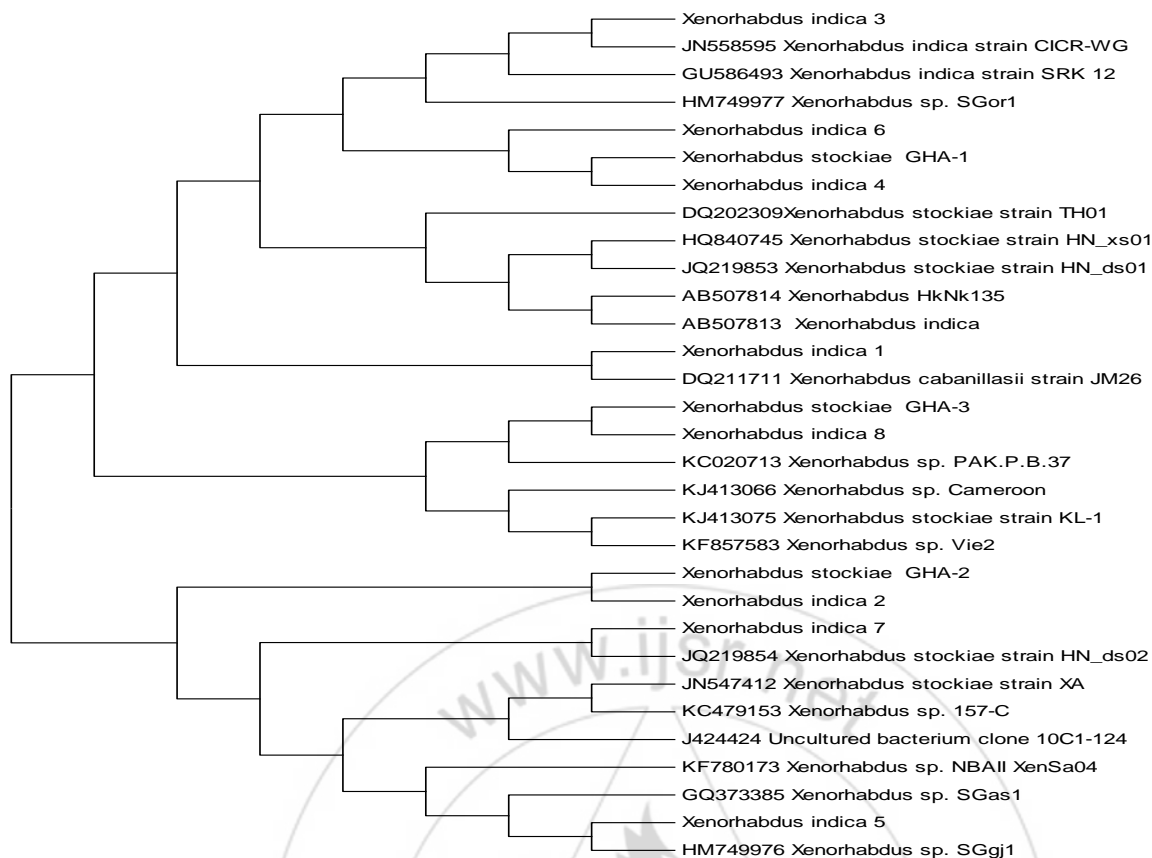
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District	Village	Crop	Nematode Host	Bacteria Species	Isolates Code
Ghaziabad	Kushliya	Cauliflower	<i>S. saimkayai</i>	<i>X.stokiae</i>	<i>X.stokiae</i> GHA-1
		Chili	<i>S. saimkayai</i>	<i>X.stokiae</i>	<i>X.stokiae</i> GHA-2
	Dasna	Radish	<i>S. saimkayai</i>	<i>X.stokiae</i>	<i>X.stokiae</i> GHA-3
Meerut	Nabipur	Chili	<i>S.thermophilum</i>	<i>X.indica</i>	<i>X.indica</i> -1
	Satli	Cauliflower	<i>S.thermophilum</i>	<i>X.indica</i>	<i>X.indica</i> -2
	Dabarsi	Radish	<i>S.thermophilum</i>	<i>X.indica</i>	<i>X.indica</i> -3
	Shobhapur	Sugarcane	<i>S.thermophilum</i>	<i>X.indica</i>	<i>X.indica</i> -4
		Radish	<i>S.thermophilum</i>	<i>X.indica</i>	<i>X.indica</i> -5
	Dhldi	Cauliflower	<i>S.thermophilum</i>	<i>X.indica</i>	<i>X.indica</i> -6
		Radish	<i>S.thermophilum</i>	<i>X.indica</i>	<i>X.indica</i> -7
	Atta chidoli	Sugarcane	<i>S.thermophilum</i>	<i>X.indica</i>	<i>X.indica</i> -8

Isolated symbiotic bacteria survey data from Ghaziabad and Meerut.





Phylogenetic relationship of *Steinernema* species based on ITS region by Neighbour-joining method.

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