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Isolation and molecular characterization of symbiotic bacterial isolates associated with entomopathogenic nematodes in agro climatic zone 5 of Karnataka

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Abstract

The investigation was carried out to study the biochemical characterization of symbiotic bacteria associated with entomopathogenic nematodes isolated from agro climatic zone 5 of Karnataka. Twenty two entomopathogenic nematodes were isolated by insect bait method using fifth instar larvae of *Galleria mellonella*. Twenty symbiotic bacteria were isolated from these entomopathogenic nematodes and named based on the source place. These isolates were identified based on microscopic observation, biochemical and physiological characters like Gelatin liquefaction test, Catalase test, Lactose fermentation test, Urease test, Motility test and Colony morphology studies on Nutrient agar, NBTA and Mac Conkey agar media. The diversity of these isolates was characterized by using RAPD markers. Primer screening was carried out using 13 decamer random primers, out of which 7 primers were detected for RAPD analysis. A total of 44 bands were scored out of which 42 bands were found to be polymorphic. Statistical analysis of RAPD data clearly depicted that all the 20 symbiotic bacterial isolates formed four major clusters. The percent polymorphism observed in the isolates was 95.45% which is quiet high and suggests that the symbiotic bacterial population exhibit high genetic diversity.

Keywords: *Galleria mellonella*, entomopathogenic nematodes, RAPD, biochemical analysis and symbiotic bacteria.

INTRODUCTION

Entomopathogenic nematodes (EPNs) are soil-inhabiting, lethal insect parasitoids that belong to the phylum Nematoda, commonly called roundworms. EPNs live inside the body of their host, and so they are designated endoparasitic. They infect many different types of soil insects, including the larval forms of butterflies, moths, beetles, and flies, as well as adult crickets and grasshoppers. EPNs have been found in all inhabited continents and a range of ecologically diverse habitats, from cultivated fields to deserts. The most commonly studied genera are those that are useful in the biological control of insect pests, the Steinernematidae and Heterorhabditidae [1]. Bacteria of the genera *Photorhabdus* and *Xenorhabdus* form a mutually beneficial symbiotic complex with the EPNs in the familiy *Heterorhabditidae* and *Steinernematidae* respectively, which are able to infect, kill and reproduce in many insect species. The life cycle of *Photorhabdus* and *Xenorhabdus* begins and ends with the colonization of the intestinal tract of a soil-dwelling and non-feeding stage of the nematode known as the infective juvenile. During this life cycle, *Photorhabdus* and *Xenorhabdus* must successfully accomplish three distinct roles: (i) rapid killing of insects, (ii) production of nutrients from the insect cadaver to facilitate growth and development of the nematode, and (iii) colonization and growth within the infective juvenile (IJ) stage of the nematode [2].

Genetic diversity can be estimated at molecular level. Randomly amplified polymorphic DNA (RAPD) technique has been used for typing and identification of number of closely related species of bacteria and assessment of genetic relationships. RAPD analysis is faster, technically less demanding and more economical than the other genomic typing methods like RFLP and AFLP. Unlike conventional PCR data on DNA sequence of the organisms are not a pre-requisite for RAPD analysis [3].Further, this technique elucidates the biodiversity in a group of isolates [4]. Hence the biodiversity of Symbiotic bacteria isolated from agroclimatic zone 5 of Karnataka were checked using RAPD analysis.

MATERIAL AND METHODS

Isolation of Entomopathogenic nematodes

Isolation of EPNs was done using *Galleria mellonella,* a host susceptible to EPNs by baiting method described by Bedding and Akhurst [5]. Small plastic vials of 50 ml capacity with wire mesh on both sides and last instar larvae of *G. mellonella* were placed in the pits dug at a depth of 15 cm in different locations of agro climatic zone 5. The nematode traps were harvested at 4, 7 and 12 days intervals to know the percent infectivity of *G. mellonella* larvae by EPNs. The infective juveniles collected from White's trap were confirmed by re-infecting nematode culture to last instar larvae of *G. mellonella*.

Isolation of symbiotic bacteria

Infected larvae were dissected and a drop of haemolymph from insect cadaver was streaked on sterile plated NBTA medium. The primary form of bacteria absorbs bromothymol blue dye and appears blue. Single colony of the bacterium stained blue were then

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selected and streaked on to new plates of NBTA medium and sub cultured continuously until colonies of uniform size and morphology were obtained.

Identification of symbiotic bacteria

The cultures so isolated were characterized through a number of morphological and physiological tests for identification of symbiotic bacteria[6,7].

Morphological Characterization
Colony morphology on different media

a) NBTA media: Each isolate was streaked on Petri plate containing NBTA medium and incubated for 4 days. Colonies were observed for absorption of bromothymol blue dye from the media and formation of typical bluish green colonies.

b) Mac Conkey agar: Each isolate was streaked on Petri plates containing Mac Conkey agar media and incubated for 5 days.

c) Nutrient agar: Each isolate was streaked on petri plate containing nutrient agar medium and incubated for 2 days.

Microscopic observation: The Symbiotic bacteria isolates were studied for cell morphology and gram reaction.

Biochemical tests: The biochemical studies viz., Gelatin liquefaction test, Catalase test, Lactose fermentation test, Urease test and Motility test were conducted.

Gelatin liquefaction test: Petriplates containing gelatin agar were spotted with overnight grown bacterial culture and incubated at 30^0C for 3 days. The plates were then flooded with 12% mercuric chloride ($HgCl_2$) solution and allowed to stand for 20 minutes. And observed for clear zones around the growth of bacterium to indicate gelatin liquefaction.

Catalase test: Nutrient slants were inoculated with bacterial cultures and incubated at 30^0C for 24 h. After incubation these tubes were flooded with 1ml of 3% hydrogen peroxide (H_2O_2) and observed for gas bubbles. Production of gas bubbles was scored positive for catalase test.

Urease test: Test tubes containing urease broth were inoculated with bacterial cultures and incubated at 30^0C for 24 h. Change of colour from yellow to red is positive for urease test.

Motility test: Motilty test medium were inoculated with bacterial cultures and incubated at 37^0c for 24-48 h. Appearance of dispersed red colour growth is positive for motility test.

Lactose fermentation test (Acid gas production): Bacterial isolates were tested for acid and gas production by inoculating 5 ml of the sterile lactose broth in test tubes containing Durham's tube. The tubes were incubated for seven days at 30^0C. Accumulation of gas in these Durham's tube is taken positive for gas production and change in colour of the medium is taken as positive for acid production.

RAPD analysis
DNA extraction protocol

DNA extraction protocol was followed was according to Sambrook *et al.,* [8] (1989).The DNA was dissolved in TE buffer and stored at 4° C.

PCR amplification conditions

PCR reactions were performed in a final volume of 25 µl containing 30 ηg of template DNA, 0.75 µl of 2mM dNTPs each, 2.5µl of 10X taq buffer,0.36µl 1 unit of *Taq* DNA polymerase, 3 µl of 10 pico mole Primer. Amplifications were achieved in MWG-Biotech primus thermocycler with the program consisting initial denaturation of 94 °C for 3 min followed by 45 cycles each consisting of denaturation at 94 °C for 1 min, primer annealing temperature at 37^0 for 1 min, primer extension at 72 °C for 3 min, and a final extension of 72 °C for 10 min. These reactions were repeated to check the reproducibility of the amplification.

Selection of primers

To choose the RAPD primers that can amplify informative sequences, Primer screening was carried out using DNA obtained from the Symbiotic bacterial isolates.

Table 1. RAPD primers with sequences chosen for analysis

Sl.No	Primer No.	Sequence
1	OP-Q 1	5'-GAG AGC CAA C-3'
2	OP-Q 2	5'-GTT TCG CTC C-3'
3	OP-Q3	5'-GTA GTC ATA T-3'
4	OP-Q 4	5'-AAG AGC CCG T-3'
5	OP-Q 5	5'-GGC TGC TGG C-3'
6	OP-Q 6	5'-CCC GTC AGC A-3'
7	OP-Q 7	5'-GAA CGG ACT C-3'
8	OP-Q 8	5'-GGT GCG GGA A-3'
9	OP-Q 9	5'-TTG GAG GGC A-3'
10	OP-Q 10	5'-CTT CCG TCA A -3'
11	OP-Q 11	5'-TGC TCT GCC C-3'
12	OP-Q 12	5'-GGT GAC GCA G-3'
13	OP-Q 13	5'-TCG CTG GGA C-3'

Analysis of RAPD data

The bands were manually scored '1' for the presence and '0' for the absence and the binary data were used for statistical analysis. The scored band data (presence or absence) was subjected to cluster analysis using STATISTICA.

RESULTS AND DISCUSSION

Isolation and Identification of Symbiotic bacteria from EPNs

Twenty isolates were isolated from EPNs and named based on the source place details provided in [Table- 2].

Colony morphology studies on different media:

a) **NBTA media**: All symbiotic bacterial isolates absorbed bromothymol blue dye from the media and formed characteristic blue colonies on NBTA media.

b) **Mac Conkey agar:**: All symbiotic bacterial isolates absorbed neutral red from MacConkey agar media and formed characteristic brown or red colonies.

c) **Nutrient agar**: All symbiotic bacterial isolates formed characteristic buff or cream colored colonies on nutrient agar media.

Microscopic observation :Symbiotic bacterial isolates were further examined for their gram reaction and shape. Characteristically all the isolates were gram negative and rod shape.

Table 2: List of Symbiotic Bacteria isolated from EPNs of agro climatic zone 5 of Karnataka

Sl.No	Isolates	Location	Crop
1	HORT1	Horticulture	Grapes
2	HORT2	Horticulture	Grapes
3	KPR1	Kanakapura	Banana
4	KPR2	Kanakapura	Banana
5	KPR3	Kanakapura	Fodder
6	KPR4	Kanakapura	Fodder
7	RMG1	Ramanagara	Paddy
8	RMG2	Ramanagara	Fodder
9	RMG3	Ramanagara	Mulberry
10	EXP1	Experimental plots, GKVK	Groundnut
11	EXP2	Experimental plots, GKVK	Groundnut
12	EXP3	Experimental plots, GKVK	Green gram
13	HEB1	Hebbal	Maize
14	HEB2	Hebbal	Maize
15	HEB3	Hebbal	Maize
16	HEB4	Hebbal	Maize
17	BGR	Botanical garden	-
18	CHK1	Chikballapur	Maize
19	CHK2	Chikballapur	Paddy
20	TUM1	Tumkur	Paddy

Biochemical and physiological characters

Gelatin liquefaction: All isolates were found positive to gelatin liquefaction as indicated by the production of yellowish or bluish green fluid on the surface of gelatin agar medium.

Catalase test: All isolates were found negative for catalase test characterized by the absence of evolution of bubbles.

Urease test: All isolates were found positive for urease test as indicated by the change of colour from yellow to red.

Motility test: All isolates were found positive for motility test indicated by the appearance of dispersed red colour bacterial growth in the motility test medium.

Lactose fermentation test (Acid gas production): All Bacterial isolates were found positive for lactose fermentation test indicated by the change in colour of the medium to yellow and appearance of gas bubbles in the Durham's tube.

Table 3. Biochemical and physiological characters of symbiotic bacterial isolates

Sl.No	Biochemical tests	Result
1	Lactose fermentation	+
2	Gelatin liquefaction	+
3	Catalase	-
4	Urease	+
5	Motility	+

RAPD characterization

For RAPD characterization, a total of 13 primers were used for preliminary screening. Finally only 7 primers producing sharp bands were selected for fingerprinting and diversity analysis of symbiotic bacterial isolates. A total of 44 bands were scored out of which 42 bands (95.45%) were polymorphic. Similar results were obtained by Dharmender Kumar [9] who carried out RAPD analysis in seventy isolates of *Bacillus thuringiensis* isolated from cotton fields,

in their study different random decamer primers were used for RAPD amplification, that generated a total of 1935 fragments; among which 1865 (96.38%) were polymorphic and 68 monomorphic (3.51%).

In our study the number of bands scored for each primer varied from 1 to 9 with an average of 6.28 bands per primer. Out of 44 amplification bands, 2 bands (4.54%) were monomorphic, 14 bands (31.81%) were unique and 28 bands (63.63%) were shared polymorphic, which were informative in revealing the relationship among the bacterial isolates [Table-4].

Cluster analysis and genetic dissimilarity matrix of 20 symbiotic bacterial isolates

The Cluster analysis based on 44 RAPD bands revealed that the twenty symbiotic bacteria isolates examined, clustered at a linkage distance of about 4 units on the dendrogram with isolate of HORT1 and isolate of CHK2 spanning the extremes. The dendrogram [Fig 1] has clearly depicted that all the 20 symbiotic bacteria isolates formed four major clusters. Among the four major groups, isolates HORT1, HORT2, HEB1, HEB2 and HEB4 formed the first group, isolates BGR, EXP1, EXP2, HEB3, KPR3 and RMG3 formed the second group, isolates RMG1, RMG2, KPR1, KPR2, KPR4 and EXP3 formed the third group and isolates CHK1,CHK2 and TUM1 formed the fourth group. Analysis of the clustering pattern indicates that isolates from geographically adjacent zones more closely related than isolates from farther away zone. Similar result was also obtained by Dharmender Kumar [9] carried out RAPD analysis in seventy isolates of *Bacillus thuringiensis* isolated from cotton field.

Table 4. Oligonucleotide primers that showed genetic variation among the Symbiotic bacterial isolates

Primers	No. of amplified fragments	No. of polymorphic bands		No. of Monomorphic bands
		shared	unique	
OP-Q 4	7	5	2	0
OP-Q 5	7	5	2	0
OP-Q 6	9	5	4	0
OP-Q 7	6	5	1	0
OP-Q 9	2	0	1	1
OP-Q 11	8	6	2	0
OP-Q 12	5	2	2	1
Total	44	28	14	2
Percentage	100	63.63	31.81	4.54

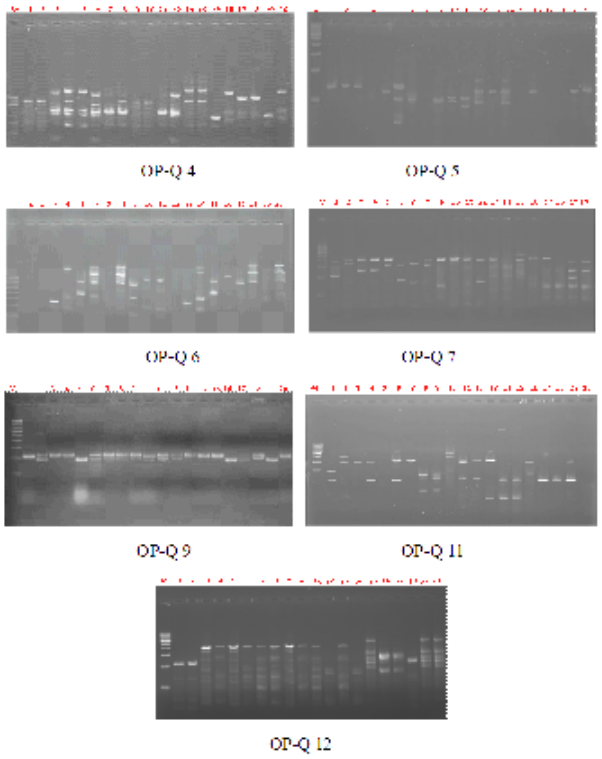

Plate 1. RAPD GEL profile of symbiotic bacterial isolates generated using 10-mer random primers (M: marker lane and lane 1 to 20 represent isolates from zone 5)

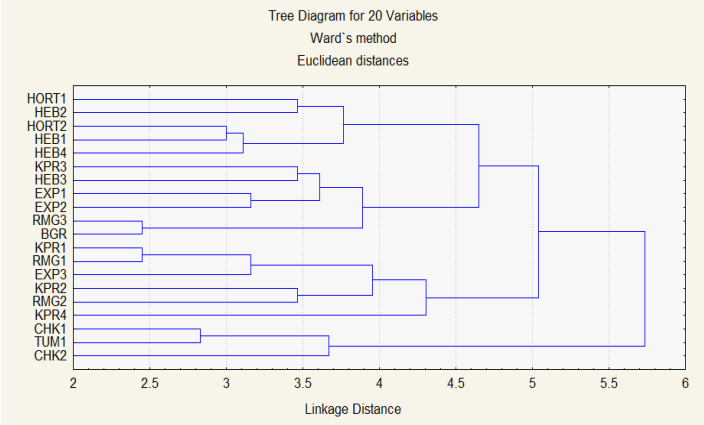

Fig 2. Dendrogram based on RAPD profile of 20 symbiotic bacteria isolated from agro climatic zone 5 of Karnataka

CONCLUSION

Symbiotic bacteria is one of best alternate to *Bacillus thuringiensis* .widespread use of *Bt* technology could eventually lead to resistance among insects. The genome of *Photorhabdus luminescens* with its many insect toxins now provides some options. Sometimes its good to have toxins from two organisms, because if you get resistance with one you can try the other.

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