MOLECULAR IDENTIFICATION OF A NEW SYMBIOTIC BACTERIA *ACHROMOBACTER* ASSOCIATED WITH ENTOMOPATHOGENIC NEMATODES

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Abstract : Entomopathogenic nematodes (EPN) lead a symbiotic association with specific enterobacteria. The nematodes and its specific symbiont are highly pathogenic to a number of insect pests and are being used as biological control agents, worldwide. While working on EPNs, presence of a number of native nematodes belonging to the family Rhabditidae having ability to enter and kill a large number of insect pests in the laboratory within 24 to 48 h of inoculation similar to the EPNs were noticed. Detailed study carried out in our lab indicated that they are associated with specific enterobacteria, which are responsible for the killing action. The bacteria lead a symbiotic association with the nematodes while being highly pathogenic to insect pests. About 65 isolates of such nematodes collected from Kerala, Karnataka, Tamil Nadu, Andhra Pradesh, Bihar, Orissa, New Delhi and Assam are being maintained alive on *Galleria mellonella* larvae reared on artificial diet. In the present study we have isolated the symbiotic bacteria from surface sterilized infective juveniles of entomopathogenic nematode isolate KO collected from Kovvur, Andhra Pradesh. DNA extraction of these bacteria were carried out and the 16S rDNA fragment was amplified using universal primer. The PCR product was cloned into pGEM-T® vector. The pGEM-T® cloned PCR products were transformed into *E.coli* (strain DH5α) cells. Transformants were selected by blue/white screening and confirmed by colony PCR, plasmid DNA isolation and its restriction. The selected clones were sent to Delhi university south campus for sequencing. Oligonucleotide sequences obtained from the sequencing reactions were compared with other available sequences in the NCBI GenBank (www.ncbi.nim.nih.gov/Entrez). The nBLAST search showed that the symbiotic bacteria isolated from entomopathogenic nematode isolate KO has 99 % identity with *Achromobacte*r sp. MT-E3 16S ribosomal RNA gene, partial sequence (Accession No. EU727196). The sequences obtained in this study have been assigned in the NCBI GenBank under the accession number HQ200411.

IndexTerms **- Entomopathogenic nematodes, 16S ribosomal RNA gene sequence, Polymerase chain reaction (PCR), *E.coli*, NCBI, *Achromobacte*r sp.**

1. INTRODUCTION

A comparison of the genomic sequences of bacterial species showed that the 16S rRNA gene is highly conserved within a species and among species of the same genus, and hence can be used as the new gold standard for the speciation of bacteria. A number of studies had been brought on the phylogenetic status of bacteria associated with EPN using 16S rRNA gene sequencing and cluster analysis. Enright *et al*. (2003) reported a novel species, *Paenibacillus nematophilus* from *Heterorhabditis* sp. based on sequencing of 16S rRNA. Clarridge (2004) studied the impact of 16S rRNA gene sequencing for the phylogeny and taxonomy of bacteria. In this study I characterized molecularly the symbiotic bacteria isolated from the EPN isolate collected from Kovvur, Andhra Pradesh.

2. RESEARCH METHODOLOGY

2.1. Isolation of Bacteria from infective juveniles

Infective juveniles (IJs) of nematodes (30 nos.) were transferred to 2 ml distilled water, treated with streptomycin (5000 units/ml) solution for one hour for surface sterilization. The nematodes were triple rinsed in sterile distilled water (Akhurst, 1980) and transferred into a micro tube having 2 ml nutrient broth (beef extract 3 gm and peptone 5 gm in 1 litre of distilled water). It was then kept in a vortex shaker for 24 hr. The solution was then streaked on to nutrient agar plates (Woodring and Kaya, 1988) and kept at room temperature for 24 hr.

2.2. Isolation of Primary colonies of bacteria

Nutrient agar (NA) of 100 ml was prepared and 0.0025 gm Bromo Thymol blue (BTB) was added and autoclaved at 121ºC for 15 min. Just before pouring the medium Triphenyl Tetrazolium Chloride (TTC) (0.04 gm) was added. Bacteria were streaked on the

plates and incubated the plates at 37ºC. The isolates were examined for the main phenotypic characteristics of the genus *Xenorhabdus*, using the methods of Boemare and Akhurst (1988). The bacterial isolates were maintained on nutrient agar slants at 5ºC.

2.3. Molecular characterization of symbiotic bacteria

2.3.1. Extraction of DNA

Qiagen DNeasy kit was used to extract DNA from cells harvested from 24 hr nutrient broth culture.

2.3.2. PCR amplification of 16S rDNA of symbiotic bacteria

The prokaryote-specific primers of Fischer-Le Saux *et al*. (1999) were used in PCR amplification of the 16S rRNA gene. The PCR was performed in a 25 µl reaction mixture having 2.5 µl of 10x Taq buffer A (Containing 15 mM $MgCl_2$), 0.5 µl 10 mM dNTP's (2.5 mM each), 1.0 µl of each primer (20 ngm), 2 µl of template DNA, and 0.25 µl of (1U) Taq DNA polymerase and 17.75 µl of sterile distilled water. The reaction was carried out in an Eppendorf thermal cycler (Eppendorf AG, Hamburg, Germany) with the thermal cycle programme of 92°C for 2 min, 10 sec (initial denaturation), 30 cycles with 94°C for 1min, 10 sec (denaturation), 49°C for 30 sec (annealing), 72°C for 2 min (extension) and final extension at 72°C for 10 min. The amplified products were resolved on a 1.5% agarose gel containing 0.5 mg ml^{-1} ethidium bromide. The DNA bands were visualized under UV transilluminator and documented through Gel Doc System (Alpha imager, Alpha Innotech, USA). 1 Kb DNA ladder (Bangalore Genei, India) was used for determining the size of the amplicon.

2.3.3. Cloning and Sequencing

The amplified PCR products were purified using the Gel Extraction Kit (QIAGEN) and the product was cloned into the pGEM-T Easy vector (Promega, USA) following the manufacturer's protocols. The *E.coli* strain DH5α was transformed with the ligated mix and the resulting recombinant clones were selected on LA medium containing Ampicillin and X-gal/IPTG (Sambrook and Russal, 2001) and confirmed by colony PCR, plasmid DNA isolation and its restriction. The clones were then subjected to sequencing in Delhi University South Campus. The sequencing was performed to both directions using T7 and SP6 primers.

3. RESULTS

The PCR amplification of the 16S rDNA of the bacteria with the primers 16SF and 16SR at an annealing temperature of 49°C yielded a fragment of approximately 1500 bp (Fig.1). The nBLAST search showed that the symbiotic bacteria isolated from entomopathogenic nematode isolate KO has 99% identity with with *Achromobacter* sp. MT-E3 16S ribosomal RNA gene, partial sequence (Accession No. EU727196). The sequences obtained in this study have been assigned in the NCBI GenBank under the accession number HQ200411.

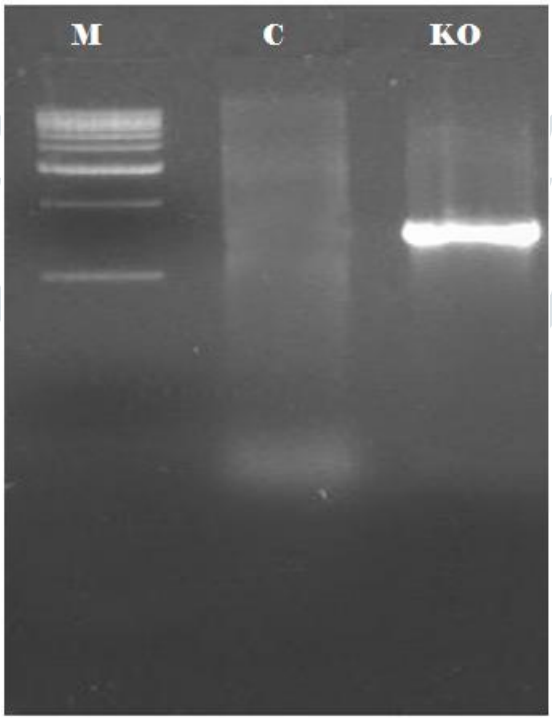

Fig.1. PCR Amplification of 16S rDNA of Symbiotic bacteria from Entomopathogenic nematode isolate KO

Fig.2. Selection of recombinant clones on LA medium containing ampicillin and X-gal/IPTG

4. DISCUSSION

Molecular methods can be employed to determine diversity among bacteria or used for rapid identification of a bacterium in question so as to avoid laborious phenotypic characterization (Adams *et al*., 2006). In the present study, the bacterial strain isolated from entomopathogenic nematodes (EPN) was investigated by molecular means including PCR and 16S rDNA sequence analysis

The PCR amplification of 16S rDNA is a practical molecular technique that is free of most subjective interpretation and allows efficient identification of symbiotic bacterial strains to the species level by genetic methods regardless of the phase status of the bacterium. This relatively simple method is much easier and less time-consuming than DNA-DNA hybridization. It enables bacterial detection with only minimal amounts of genomic DNA and with simultaneous analysis of several isolates on a single gel. Presently, bacterial species identification using the 16S rDNA-based method is the most widely accepted method as large public-domain sequence databases is available for comparison (Maidak *et al*., 1996).

Molecular approaches to the identification of bacteria show promising results. The amplification of 16S rDNA of any bacterial species is possible without prior cultivation when broad range PCR primers targeted to highly conserved regions are applied. The comparison of amplified and sequenced 16S rDNA sequences with sequences of known bacteria in 16S rDNA databases facilitates a subsequent phylogenetic identification. Furthermore, because the number of 16S rDNA sequences in public databases is increasing day by day, a better identification of so far unknown bacteria may be possible in the future. Direct sequence determination of 16S rRNA gene fragments represents a highly accurate and versatile method for identification of bacteria to the species level, even when the species in question is notoriously difficult to identify by biochemical means.

5. ACKNOWLEDGEMENT

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6. REFERENCES

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