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Bacterial Diversity in Fluoride - Contaminated Soils Using 16S rDNA Sequencing

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Abstract: ***In the unique Kuttanad region of Kerala, India, renowned for its low - lying paddy fields and intricate waterways, this study investigates molecular microbial diversity in fluoride - contaminated soils. Key to this exploration is the molecular technique 16S rDNA sequencing. Using these molecular techniques, specific bacterial species such as Pseudomonas plecoglossicida (KTB1), Bacillus subtilis (KMB2), and Pseudomonas putida (KMNB3) were revealed within these soils. The application of molecular techniques emerges as a powerful tool for understanding specific microbial diversity in challenging environmental conditions.***

Keywords: 16S rDNA sequencing, molecular technique

1. Introduction

A number of approaches have been developed to study molecular microbial diversity. These include DNA reassociation, DNA - DNA and mRNA: DNA hybridization, DNA cloning and sequencing and other PCR - based methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), ribosomal intergenic spacer analysis (ARISA) (Kirk et al., 2004).

In the realm of microbial diversity exploration, analysis of the 16S rDNA molecule or its corresponding gene (16S rDNA) is by far the most widely used method in the last decade (Amann et al., 1995). Its significance lies in its dual nature, featuring both highly conserved and variable regions, making it the go - to marker for taxonomic identification and evolutionary insights. The sequencing of the 16S rDNA gene unveils the taxonomic identity of bacterial and archaeal species, allowing for classification at various hierarchical levels. Moreover, it forms the backbone of phylogenetic analysis, helping construct evolutionary trees that elucidate the relationships among microbial species. In the world of metagenomics, this gene takes centre stage, as it empowers researchers to delve into the intricate web of microbial communities without the need for cultivation. Of the 16S rDNA - based methods used for studying complex microbial populations, denaturing gradient gel electrophoresis (DGGE) has received wide attention and has been successfully applied to several natural habitats (Murray et al., 1996; Jensen et al., 1998)

2. Materials and Methods

2.1 Site description

Kuttanad is a unique and captivating region situated in the southern Indian state of Kerala. The site of is characterized by a vast expanse of lush, low - lying paddy fields, crisscrossed by an intricate network of serene backwaters, rivers, and canals. Kuttanad's iconic paddy fields, often hailed as the "Rice Bowl of Kerala, " epitomize the region's rich agricultural legacy. The unique wetland farming system, characterized by fields below sea level and regulated water levels through bunds, is a testament to the region's adaptability and traditional knowledge. The soil, predominantly composed of alluvial clay and enriched with organic matter, occupies a central role in the region's prosperity. At the same time, the unique geological composition of Kuttanad, including the prevalence of fluorapatite in Tertiary sediments, contributes to the elevated levels of fluoride in the water samples (Raj and Shaji, 2017), or from the interactions involving agricultural inputs such as fertilizers, pesticides, and various chemicals, as proposed by Annadurai et al in 2014.

2.2 Soil collection

15 locations of Kuttanad rice fields were selected for soil sampling during the fallow period. For studying molecular characterization, soils from three locations which had a low, medium and high F - concentration (Roshni and Harikumar, 2021) were chosen. Three predominant bacterial isolates of *Pseudomonas* and *Bacillus* (KTB1, KMB2 and KMNB3) identified based on morphological, physiological and biochemical characteristics were subjected to molecular characterization. The isolates were maintained on agar slants at 4^0C as stock cultures.

2.3 Molecular identification of bacterial isolates by16S rDNA gene sequencing

DNA isolation and PCR amplification
Genomic DNA was extracted from the bacterial culture using the Sigma Aldrich DNA extraction kit. The purity and concentration of the extracted DNA were assessed through 0.8% (wt/vol) agarose gel electrophoresis and quantified using a UV - Vis Spectrophotometer (Systronics, India) at 260 nm and 280 nm, respectively. The concentrations of the extracted DNA were then adjusted to a final concentration of 10 µg/ml.

Amplification of 16S rDNA fragments were performed by using thermocycler (SureCycler 8800, Agilent, USA) with universal primers 8F: 5’ - AGAGTTTGATCMTGG - 3’ and 1492R: 5’ - ACCTTGTTACGACTT - 3’ primer pair. The

PCR reaction mixture contained 20 ng of DNA template, 1.5 mM $MgCl_2$, 10 mM dNTP mixture and 10 pM of each primer and 5 U of *Taq* DNA polymerase with reaction mixture supplied by the manufacturer in a total volume of 25 µl. Reaction mixture was first denatured at 95^0C for 5 min, followed by denaturation at 94^0C for 30 s, annealing at 52^0C for 30 s and extension at 72^0C for 45 s. Amplification was completed by a final extension step at 72^0C for 10 min and the reaction was carried out for 30 cycles. PCR products were run on 1% agarose gel in 1×TBE buffer stained with ethidium bromide and were visualized with UV transilluminator. Amplified PCR products were purified using column purification as per manufacturer's guidelines.

Nucleotide sequencing

The purified PCR products were directly sequenced using a DNA analyzer (model ABI3730xl, Applied Biosystems, USA). Primers used for this purpose were same as for the PCR.

Phylogenetic analysis

The 16S rDNA sequences were initially analyzed using BLAST program (www.ncbi. nlm. nih. gov/blast/blast. cgi). Multiple sequence alignments of approximately 900 base pair sequences were performed using BioEdit software version 7.2. Phylogenetic tree was constructed using the neighbor joining method (Saitou and Nei, 1987). The 16S rDNA sequences determined in the study are deposited in the Gen Bank databases, under the accession numbers *MW092513*, *MW092560* and *MW092709*.

3. Result and Discussion

Molecular identification of bacterial isolates from F - contaminated soils

In (0.8%) agarose gel electrophoresis ~1300bp band was obtained by PCR amplification (Fig.1). BLAST analysis of the sequence data revealed high similarity (99%) with bacteria *Pseudomonas plecoglossicida* (KTB1), 100% similarity with *Bacillus subtilis* (KMB2) and 99.89% (Fig.2) similarity with *Pseudomonas putida* (KMNB3). The nucleotide sequences were deposited in the Genbank database under the accession numbers MW092513 (*Pseudomonas plecoglossicida*), MW092560 (*Bacillus subtilis*) and MW092709 (*Pseudomonas putida*).

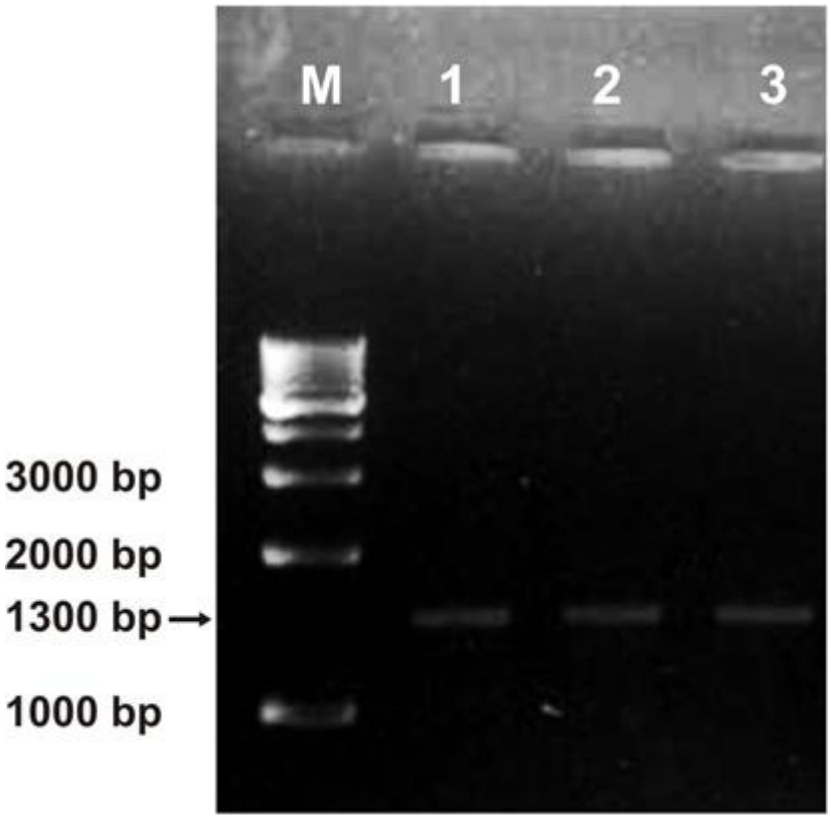

Figure 1: PCR amplification of 16S rDNA: Lane M= 10 Kb marker, lanes 1, 2 & 3 represent three different bacterial isolates viz. KTB - 1, KMB - 2 and KMNB - 3 In the gel, size of the amplified PCR product was 1300bp.

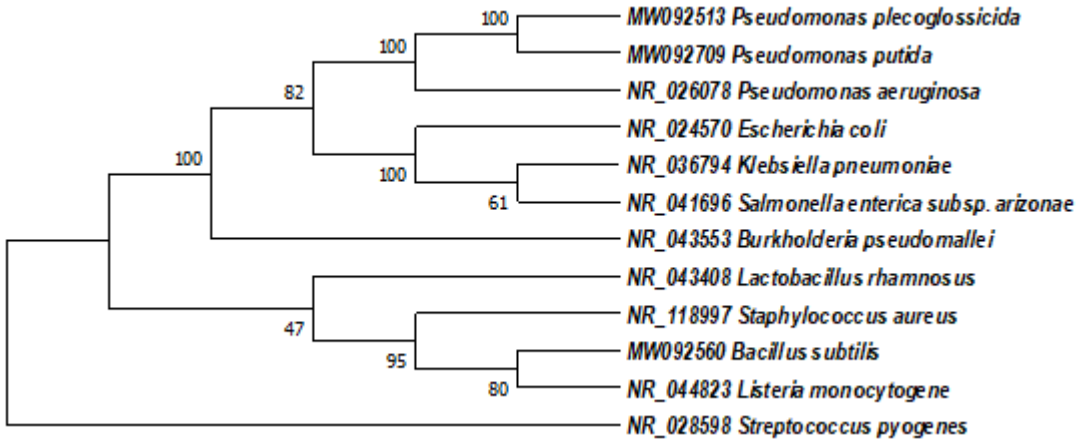

Figure 2: Phylogenetic tree of bacterial sequences obtained from F ˉ contaminated soil

Although conventional phenotypic methods are relatively inexpensive and allow identification of most commonly encountered microorganisms, certain groups of microorganisms are difficult to identify and require special equipments and expertise.

16S rDNA sequencing represents a universal technology that theoretically provides solution to these problems (O'Donnel and Görres, 1999).16S rDNA sequencing is particularly useful in identifying unusual bacteria and actinomycetes that are difficult to identify by conventional methods providing genus identification in >90% of cases, and identification of 65 - 83% of these at the species level (Drancourt et al., 2000; Mignard and Flandrois, 2006).

In the present study however, 16S rDNA sequencing has been performed to unravel the correct identity of bacterial and actinomycete isolates from F ˉ contaminated soils of Kuttanad.16S rDNA gene sequence data on phylogenic analysis showed close matching of the isolates with the type strains of bacteria belonging to the genera *Pseudomonas* and *Bacillus* sp. Similar was the case with actinomycete isolates which showed lineages with the type strains *Streptomyces* sp. indicating the suitability of 16S rDNA sequencing as a powerful marker in evaluating bacterial composition/diversity in F ˉ contaminated soils.16S rDNA gene sequencing was applied by several researchers for the identification of bacteria (Felske and Akkermans 1998; Deshmukh et al., 2011) and actinomycetes (Jeffrey, 2008; Isik et al., 2014) in various ecosystems including polluted sites (Mitra and Roy, 2010; Banerjee et al., 2016).

4. Conclusion

This study delves into the world of microbial diversity in the unique landscape of Kuttanad, revealing its rich tapestry of bacterial life. Molecular techniques, specifically 16S rDNA sequencing, served as powerful tools for unraveling the identities of various microorganisms thriving in fluoride - contaminated soils. The 16S rDNA sequencing unveiled the presence of bacteria primarily belonging to the Pseudomonas and Bacillus genera within fluoride - contaminated soils. Molecular techniques have paved the way for a deeper understanding of these complex organisms, especially in ecosystems where conventional methods fall short.

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