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PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogenetic Analysis of *Pseudomonas aeruginosa*

K. Amutha[1], V. Kokila[2]

[1]Associate Professor, Department of Biotechnology, Vels University, Chennai, Tamil Nadu, India

[2]Research Scholar, Department of Biotechnology, Vels University, Chennai, Tamil Nadu, India

Abstract: ***Pseudomonas aeruginosa is a gram negative bacterium isolated from soil. The isolate was cultured in a selective medium (Pseudomonas Agar base) at 37ºC for 24 hours. For species identification, Pseudomonas like organisms creates lot of problems when identified with the help of morphological and biochemical characters. However, sequencing of 16S rRNA region is a suitable technique for species identification. The amplified product of 16S rRNA was submitted to NCBI database. Amplification of 16S rRNA gene region, and new sets of primer pairs were designed by NCBI database search tool. To study phylogenetic relationship between various strains of Pseudomonas aeruginosa have often been based on sequencing of 16S rRNA gene region. Distance tree was constructed to find out genetic similarity between the organisms. Hence gene sequencing of 16S rRNA region was a suitable technique to identify Pseudomonas aeruginosa at molecular level.***

Keywords: *Pseudomonas aeruginosa*, Amplification, 16S rRNA, Phylogenetic relationship, NCBI, BLAST

1. Introduction

The genus Pseudomonas is a gram negative, rod shaped microorganisms [1] reported to have ecological, economic and health related importance's. Some Pseudomonas species are reported to be pathogenic for plants [2, 3], opportunistic pathogens of animals or humans [4, 5, 6] and some are used for bio control agent because it exhibit plant growth promoting and pathogen suppressing functions [7, 8].

Sequencing of 16S rRNA is a molecular technique for characterization of bacteria and tools involved is to analyse the phylogenetic relationship of an organism [9]. For species identification various molecular methods has been raised. According to Busse et al 1996 [10], the molecular biological methods such as nucleic acid analysis, protein pattern or fatty acid profiles has been proved for identification of bacteria rapidly. Identification of bacteria at species level, DNA sequences at genus-specific might be widely used. According to Barrey et al 1991 [11], Jensen et al 1993 [12], Gurtler & Stanisich 1996 [13] 16S-23S rRNA intergenic spacer of the ribosomal RNA opearn (RRN) gene region is used for identification of strains and species.

The aim of this study was, sequencing of the 16S rRNA gene region of *Pseudomonas aeruginosa* isolated from soil. To design genus- specific new set of primer pairs from the NCBI database search tool and to study the phylogenetic relationship between the various strains of *Pseudomonas aeruginosa*.

2. Materials and Methods

Pseudomonas aeruginosa was isolated from the soil sample collected at Maduravoyal near chennai. It was cultured in the selective medium (Pseudomonas Agar Base) and incubated at 37ºC for 24 hours. The isolate was identified based on the morphological and biochemical characters [14]. For species identification sequencing of 16S rRNA gene region was carried out.

2.1 DNA Extraction

DNA was extracted from *Pseudomonas aeruginosa* based on the method described by Pitcher et al 2008 [15]. After extraction the DNA sample was run on 1% agarose gel at a constant voltage of 100V. The gel was examined on UV transilluminator.

2.2 PCR amplification and sequencing of 16S rRNA

16S rRNA gene region was amplified with the universal primers. For setting up PCR, the following reaction mixtures were added into the PCR tube. The reaction mixtures were 5µl of template, Primers: 1 µl of Forward primer- 27F (5' AGAGTTTGATCCTGGCTCAG 3'), 1 µl of Reverse primer- 1492R (5' TACCTTGTTACGACTT 3') [16], 6 µl of assay buffer, 2 µl of Taq DNA polymerase and 5 µl of dNTP mix (Applied Biosystems, Acme Progen Biotech (India) pvt. Ltd, Salem, Tamilnadu, India). The amplification was carried out in a thermal cycler for 40 cycles using the following reaction conditions, denaturation of DNA at 94ºC for 1 minute, primer annealing at 56 ºC for 30 seconds and primer extension at 72 ºC for 1 minute. The amplified PCR product was mixed with 5 µl of gel loading buffer. 1.5% agarose gel was casted. The samples were loaded along with 5 µl of 1kb DNA ladder (HIMEDIA, Mumbai, Maharashtra, India) as a molecular marker. The gel was run and examined on UV transilluminator to visualize the bands. PCR products were purified by using the PCR Klenzol™ (Genei, Bangalore, India) and it was sequenced with an ABI Prism 3700 DNA Analyzer (Acme Progen Biotech (India) Pvt. Ltd,, Salem, Tamilnadu, India).

2.3 Nucleotide sequence accession number and BLAST analysis

The nucleotide sequence 16S rRNA gene region data was submitted to NCBI nucleotide sequence database. Using BLAST tool, phylogentic tree, primer pairs were designed from NCBI database search tool.

3. Result

Pseudomonas aeruginosa was isolated from soil. Based on the morphological and biochemical characters it was identified as *Pseudomonas aeruginosa* (Table 1). *Pseudomonas aeruginosa* was gram negative, which shows positive result on catalase and oxidase test. Hence to identify and confirm the *Pseudomonas aeruginosa* at molecular level, 16S rRNA gene region was amplified and sequenced. Genomic DNA was extracted from *Pseudomonas aeruginosa* by the standard method. PCR amplification of 16S rRNA gene region by using universal primer, the obtained PCR product resulted in 1451 bp (Fig. 1). The sequence was submitted to NCBI database and the accession number is KC119195. By using BLAST analysis, 98 sequences of NCBI data gave 99% similarity. Phylogenetic tree generated by NCBI tool proves that this organism genetically related with other organisms (Fig. 2). NCBI primer blast tool help us to search new primer pairs to amplify 16S rRNA gene region (Table 2).

Table 1: Morphologial And Biochemical Identification Of Pseudomonas Aeruginosa

S.No	*Characteristics*	
	Morphological Identification	
1.	Gram Staining	Gram negative, Rods
2.	Colony	Small, pigmented, circular
	Biochemical Identification	
1.	Catalase	+
2.	Oxidase	+
3.	Methyl Red	–
4.	Indole	+
5.	Citrate Test	+
6.	Nitrate Test	+

+-Positive, – Negative

1 AGATTGAACG CTGGCGGCAG GCCTAACACA TGCAAGTCGA GCGGATGAAG
51 GGAGCTTGCT CCTGGATTCA GCGGCGGACG GGTGAGTAAT GCCTAGGAAT
101 CTGCCTGGTA GTGGGGGATA ACGTCCGGAA ACGGGCGCTA ATACCGCATA
151 CGTCCTGAGG GAGAAAGTGG GGGATCTTCG GACCTCACGC TATCAGATGA
201 GCCTAGGTCG GATTAGCTAG TTGGTGGGGT AAAGGCCTAC CAAGGCGACG
251 ATCCGTAACT GGTCTGAGAG GATGATCAGT CACACTGGAA CTGAGACACG
301 GTCCAGACTC CTACGGGAGG CAGCAGTGGG GAATATTGGA CAATGGGCGA
351 AAGCCTGATC CAGCCATGCC GCGTGTGTGA AGAAGGTCTT CGGATTGTAA
401 AGCACTTTAA GTTGGGAGGA AGGGCAGTAA GTTAATACCT TGCTGTTTTG
451 ACGTTACCAA CAGAATAAGC ACCGGCTAAC TTCGTGCCAG CAGCCGCGGT
501 AATACGAAGG GTGCAAGCGT TAATCGGAAT TACTGGGCGT AAAGCGCGCG
551 TAGGTGGTTC AGCAAGTTGG ATGTGAAATC CCCGGGCTCA ACCTGGGAAC
601 TGCATCCAAA ACTACTGAGC TAGAGTACGG TAGAGGGTGG TGGAATTTCC
651 TGTGTAGCGG TGAAATGCGT AGATATAGGA AGGAACACCA GTGGCGAAGG
701 CGACCACCTG GACTGATACT GACACTGAGG TGCGAAAGCG TGGGGAGCAA
751 ACAGGATTAG ATACCCTGGT AGTCCACGCC GTAAACGATG TCGACTAGCC
801 GTTGGGATCC TTGAGATCTT AGTGGCGCAG CTAACGCGAT AAGTCGACCG
851 CCTGGGGAGT ACGGCCGCAA GGTTAAAACT CAAATGAATT GACGGGGGCC
901 CGCACAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG CGAAGAACCT
951 TACCTGGCCT TGACATGCTG AGAACTTTCC AGAGATGGAT TGGTGCCTTC
1001 GGGAACTCAG ACACAGGTGC TGCATGGCTG TCGTCAGCTC GTGTCGTGAG
1051 ATGTTGGGTT AAGTCCCGTA ACGAGCGCAA CCCTTGTCCT TAGTTACCAG
1101 CACCTCGGGT GGGCACTCTA AGGAGACTGC CGGTGACAAA CCGGAGGAAG
1151 GTGGGGATGA CGTCAAGTCA TCATGGCCCT TACGGCCAGG GCTACACACG
1201 TGCTACAATG GTCGGTACAA AGGGTTGCCA AGCCGCGAGG TGGAGCTAAT
1251 CCCATAAAAC CGATCGTAGT CCGGATCGCA GTCTGCAACT CGACTGCGTG
1301 AAGTCGGAAT CGCTAGTAAT CGTGAATCAG AATGTCACGG TGAATACGTT
1351 CCCGGGCCTT GTACACACCG CCCGTCACAC CATGGGGAGT GGGTTGCTCC
1401 AGAAGTAGCT AGTCTAACCG CAAGGGGGGA CGGTTACCAC CGGAGTGATT
1451 CATGACTGGG GGTGAAGTCG TAACAAGGTA GCCTAG
//

Figure 1: Amplified 16S rRNA gene region

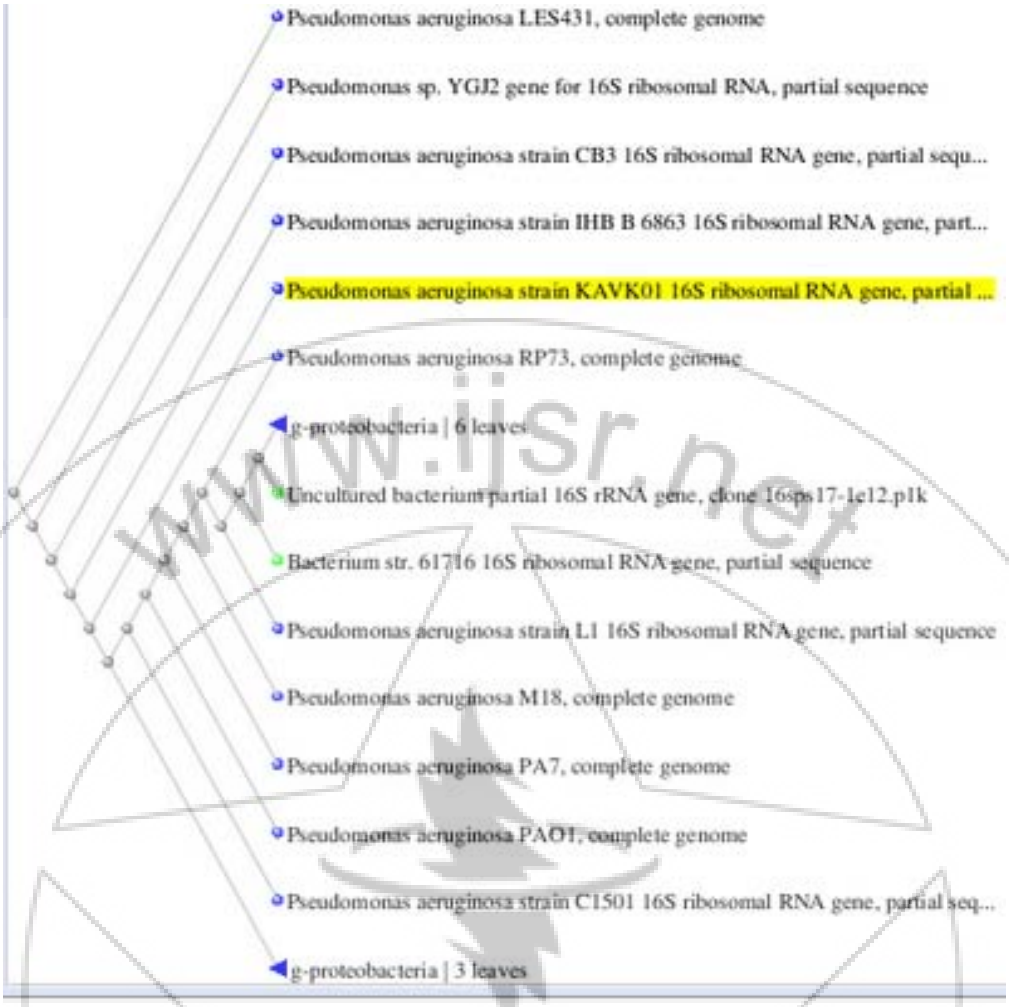

Figure 2: Distance Tree Using Blast Tool in the Ncbi Database Search Tool Of (Accession No: Kc119195) (SOURCE: http;//www.ncbi.nlm.nih.gov/blast/treeview/tree View)

Table 2: Designing of Primer Pairs Using Primer Blast Tool In The Ncbi Database Search Tool Of (Accession No: Kc119195) (Source: http://www.ncbi.nlm.nhi.gov/tools/primer-blast/primertools)

S.NO	*Primer Pairs*	*Sequence (5'->3')*	*Template Strand*	*Length*	*Product Length*
1.	Forward Primer	CGTCCTGAGGGAGAAAGTGG	Plus	20	111
	Reverse Primer	CAGTTACGGATCGTCGCCTT	Minus	20	
2.	Forward Primer	AGTTGGGAGGAAGGGCAGTA	Plus	20	237
	Reverse Primer	ATTCCACCACCCTCTACCGT	Minus	20	
3.	Forward Primer	CAAGGCGACGATCCGTAACT	Plus	20	119
	Reverse Primer	ATCAGGCTTTCGCCCATTGT	Minus	20	
4.	Forward Primer	TAAAGGCCTACCAAGGCGAC	Plus	20	414
	Reverse Primer	CCACCACCCTCTACCGTACT	Minus	20	
5.	Forward Primer	GTAGTGGGGGATAACGTCCG	Plus	20	141
	Reverse Primer	TCGCCTTGGTAGGCCTTTAC	Minus	20	
6.	Forward Primer	GGGGTAAAGGCCTACCAAGG	Plus	20	135
	Reverse Primer	GATCAGGCTTTCGCCCATTG	Minus	20	
7.	Forward Primer	GTACGGTAGAGGGTGGTGGA	Plus	20	785
	Reverse Primer	GCTACTTCTGGAGCAACCCA	Minus	20	
8.	Forward Primer	CTGCCTGGTAGTGGGGGATA	Plus	20	149
	Reverse Primer	GTCGCCTTGGTAGGCCTTTA	Minus	20	
9.	Forward Primer	ACAATGGGCGAAAGCCTGAT	Plus	20	137
	Reverse Primer	GCCGGTGCTTATTCTGTTGG	Minus	20	
10.	Forward Primer	TACGGTAGAGGGTGGTGGAA	Plus	20	781
	Reverse Primer	ACTTCTGGAGCAACCCACTC	Minus	20	

4. Discussion

In the past decade, the Pseudomonas classification had attracted more attension and it was reclassified by Brosch et al 1996, Kersters et al 1996, Palleroni 1992 [17, 18, 19]. Identification of Pseudomonas creates lot of difficulties [20, 21, and 4]. Morphologically similar species are alike to have biochemical characters. Sequence of highly conserved gene region 16S rRNA data helps us for the prediction of correct taxonomy. Our present study was carried out to sequence 16S rRNA based on PCR amplification for identification and genetic level conformation of *Pseudomonas aeruginosa*.

NCBI database provide us more information regarding nucleotide sequences. The revealed sequence accession number is KC119195 in NCBI provide us taxonomy report. Ninety eight organisms were evaluated with 99% similarity in BLAST analysis. In phylogenetic tree these two organism *Pseudomonas aeruginosa* strain IIIB B 6863 16S ribosomal RNA gene, partial sequence and *Pseudomonas aeruginosa* RP73, Complete genome were genetically very close to our strain of *Pseudomonas aeruginosa*. Primer pairs database search tool revealed that 10 new sets of primer pairs. These sets were very helpful for further sequencing of 16S rRNA species specific gene region.

5. Conclusion

The present study explained that some *Pseudomonas aeruginosa* strains are morphologically similar but different at genetic level. Hence amplification of 16S rRNA gene region is the suitable technique for identification of *Pseudomonas aeruginosa* is more accurate. By using nucleotide database search tool, analysing 16S rRNA gene sequence of *Pseudomonas aeruginosa* similarities with other strains, the phylogenetic relationship was constructed and new set of primer pairs were designed. Designing of new primers will help species level identification at molecular level in future.

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Author Profile

Dr. K. Amutha working as Associate professor in Department of Biotechnology, Vels University, Chennai, Tamil Nadu, India.

V. Kokila is a Ph.D Scholar in the Department of Biotechnology, Vels University, Chennai, Tamil Nadu, India.