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# 16S rRNA GENE ANALYSIS FOR CHARACTERIZATION OF *PSEUDOMONAS* SPP. ISOLATED FROM GROUNDNUT RHIZOSPHERE.

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**ABSTRACT:** The present study assessed 16S rRNA gene sequence analysis method as a tool for identification of fluorescent pseudomonads phenotypically difficult to identify. Using 16S rRNA gene sequencing the isolates were identified to the genus level to the species level. In this study, phylogenetic tree was constructed using complete sequence within the 16S rRNA gene. Distance tree was constructed to find out genetic similarity between the organisms. Although the phylogenetic analysis is not decisive, it is consistent with other observations, especially the capacities of the strain as a biocontrol agent. The results suggest that the JS16, JS7, JS31 and JS52 and strains are high homology with Pseudomonas fluorescens, P. Plecogloccida and P. Putida.

**Key words:** 16S rRNA, *Pseudomonas fluorescens, P. Plecogloccida*, *P. Putida,* Phylogenetic analysis

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## INTRODUCTION

Bacteria belonging to the fluorescent pseudomonads, known for the diversity of their metabolites, bio-control, bio-fertilizing activity is essential to understand their ecological role in the rhizosphere. In recent years certain fluorescent pseudomonads species, for example *Pseudomonas chloroaphis*, *P. fluorescens*, *P. veronii*, *P. putida*, have received attention even though they possess abilities to influence plant growth and development through different mechanisms (Weller, 1988; O'Sullivan and O'Gara, 1992). They are now recognised as being antagonistic to numerous soil-borne fungi (Weller, 1988; Keel *et al.,* 1996) and to seed borne fungi (Hokeberg *et al.,* 1997). Also, some strains are responsible for major plant growth promoting effects (DeFreitas and Germida, 1991; Kropp *et al.,* 1996).

Fluorescent *Pseudomonas* spp. belongs to the rRNA group I of the gamma subclass of *Proteobacteria* (Palleroni, 1993; Kersters *et al.,* 1996). In this group *Pseudomonas* spp. have been separated on the basis of their DNA homologies (Johnson and Palleroni, 1989; Gardan *et al.* 1992). This method of differentiating bacterial species has for several years been the most reliable. But now the most extensive phylogenetic studies of *Pseudomonas* spp. have been based on the 16S rRNA gene (Kersters *et al*., 1996; Anzai *et al*., 2000; Porteous *et al*., 2002). Its sequence is a miscellany of highly conserved regions of variable and hyper variable stretches that makes it suitable for PCR primer design. *Pseudomonas* 16S rRNA gene contains 1492 nucleotide positions, of which 148 are variable and 65 positions of these are present in three hyper variable regions. The region hyper variable 1 is considered one of the most variable sequences in 16S rRNAs of bacteria across the phylogenetic spectrum and is useful for differing intra generic lineages of *Pseudomonas*. As a result of such studies, many organisms classified as species of this genus have been reclassified (Kersters *et al.,* 1996).

Bacteria belonging to the genus *Pseudomonas*, data collected based on 16S rRNA genes make taxonomical studies easier, together with those in the rhizosphere. In the present paper, we successfully applied sequencing of the 16SrDNA for clarifying the classification of the isolates JS7, JS31, JS52 and JS16.

## MATERIALS AND METHODS

### Total Genomic DNA isolation from isolated strains:

Four potential bacterial biocontrol isolates were tested Phylogenetic analysis. A revised protocol (Ausubel *et al,.* 1995) was used for DNA isolation. Bacteria were grown in 5 ml LB broth 18-20 h at 28°C at a rotation speed of 200 rpm. 1.5 ml culture was centrifuged for 2 min in an eppendorf tube after discarding the supernatant, the pellet was resuspended in 576 µl TE buffer (10 mM Tris. Cl, 1 mM EDTA, pH = 8). Ten percent SDS and 20 mg/ml Proteinase K was added to make final concentrations of 5% and 0.5%, respectively. Samples were mixed thoroughly, and incubated at 37°C for 1 h. After adding 5 M NaCl to 714 mM and CTAB/NaCl (CTAB: hexadecyltrimethl-ammonium bromide, Sigma®, Sigma Chemical Co., St. Louis, MO., U.S.A) to 10%, the samples were incubated at 65°C for 10 min. Samples were extracted with equal volume of 24:1 chloroform /isoamyl alcohol and then 25:24:1 phenol /chloroform /isoamyl alcohol and centrifuged at 13.000 rpm for 10 min. The supernatant was transferred to a fresh eppendorf tube after each centrifugation. DNA was precipitated by adding 0.6 vol. cold isopropanol. After 5 min the samples were centrifuged again at 13,000 rpm for 10 min. The supernatant was discarded and the pellet was washed by 70% ethanol. The eppendorf tubes were placed upside down to let them air dry for about 30min. The pellet was resuspended in 100 µl of warm TE buffer (10 mM Tris. Cl, 1 mM EDTA, pH = 8). The DNA extracts were stored at 4°C. In order to catalyze the hydrolysis of RNA, Ribonuclease A (Sigma , St. Louis, MO, USA.) were added to 0.02 µg, and then incubated at 37°C for 30 min. DNA was precipitated again with isopropanol and resuspended in 100 µl of TE buffer as previously described. Samples were stored at –20°C.

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### DNA quantification

DNA quantification was determined by gel electrophoresis and the spectrophotometer. Agarose gel of 1.2% with ethidium bromide (0.5µg/ml) and 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) was used in the electrophoresis. The 1 kb DNA size marker was used to estimate the size of the extracted DNA. The gel was run at 150 volts for approximately 40 min. A photograph was taken under the UV transilluminator.

OD values of DNA were read at 260 nm and 280 nm using the spectrophotometer. The ratio of $OD_{260}$ to $OD_{280}$ was calculated to assess the DNA purity.

### 16s PCR amplification:

16s PCR was carried out with primers including 16s (F: AGAGTTTGATCCTGGCTCAG, R: CGG TTA CCT TGT TAC GAC TT), PCR was repeated at least twice to confirm the results. Total genomic DNA isolated from four bacterial isolates was used as template for PCR amplification. 50 ng DNA was used as template in 20 µl of reaction mixture containing 10 µM of each primer, 0.2 mM of each of the four dNTPs, (1X) *Taq* DNA polymerase buffer, 1 U *Taq* DNA polymerase . PCR cycling profile was 1 cycle at 94ºC for 5 min, 35 cycles of 94ºC for 1 min, 52ºC and 72ºC for 1 min followed by a final extension step at 72ºC for 8 min. PCR reactions amplified with water instead of genomic DNA were used as negative controls.

### Electrophoresis of amplified PCR products

Amplified PCR products were separated using Agarose gel electrophoresis in 1.0% TAE buffer and stained with 0.5 µg/ml Ethidium bromide at 60V and 1x TAE gel running buffer according to (Sambrook *et al.,* 2001). 100 bp ladder (NEB: 3231 L) was used as a DNA marker to determine the size of amplified DNA fragments. Gels were viewed on a UV-transilluminator, and then documented with the help of a gel documentation system (Biorad).

### Sequencing and BLAST search

To further confirm the positive results from PCR reactions, the desired PCR products were sent for sequencing (University Core DNA & Protein Services, University of Calgary, Calgary, AB). For the reaction with primers the desired band (470 bp) was cut from the agarose gel, and then the DNA was purified with 5M NaCl and 95% ethanol. After re-dissolution in water, the purified DNA was used as template to amplify the desired PCR product again with primers. The PCR product was checked and purified as described previously. The DNA was quantified after gel electrophoresis, by comparing with 1650 bp band of 1 kb DNA ladder. Each 12 µl premixed sample for sequencing contained 0.01 µg of template DNA per 100 bp, and 3.2 pmol primer. The results were aligned on the BLAST search at National Centre for Biotechnology Information (NCBI) using nucleotide-nucleotide BLAST (blastn) and align two sequences (bl2seq).

**Molecular phylogeny**

The reference sequences required for comparison were downloaded from the EMBL database using the site http://www.ncbi.nlm.nih.gov/Genbank. The sequences of 16s rRNA, of antagonistic Fluorescent Pseudomonads were aligned using the multiple sequence alignment program CLUSTAL W developed by Higgins *et al.,* (1992). Phylogenetic tree was constructed from the data sets of 16s gene sequence as well as from the combined nucleotide sequences of these two genes from the same strain to achieve better resolution and reliability.

## RESULTS

**Taxonomic identification and phylogenetic analyses of fluorescent Pseudomonads**

Phenotypic classification of *Pseudomonas* spp. has proved difficult as several members of the genus exhibit similar characteristics. The most extensive phylogenetic studies of *Pseudomonas* spp. have been based on the 16S rRNA gene sequence is a miscellany of highly conserved regions of variable and hyper variable stretches which undergo fewer changes than most other DNA sequences in the course of evolution that makes it suitable for PCR primer design and phylogenetic analysis (Fig. 1). Amplification of a 16S rRNA gene was approximately 1450 bp to 1550bp, from all the 4 isolates. The amplified product was sequenced and sequence of DNA fragment was compared to the sequences available in Gene Bank, NCBI. Sequence analysis of these isolates was also performed using BLAST (blastn) search tool (http://www.ncbi.nlm.nih.gov) available on the NCBI home page. It is confirmed that each isolates were related to pseudomonads. All the fluorescent *Pseudomonas* genotypes clustered with the *Pseudomonas* reference strains (Fig. 2).

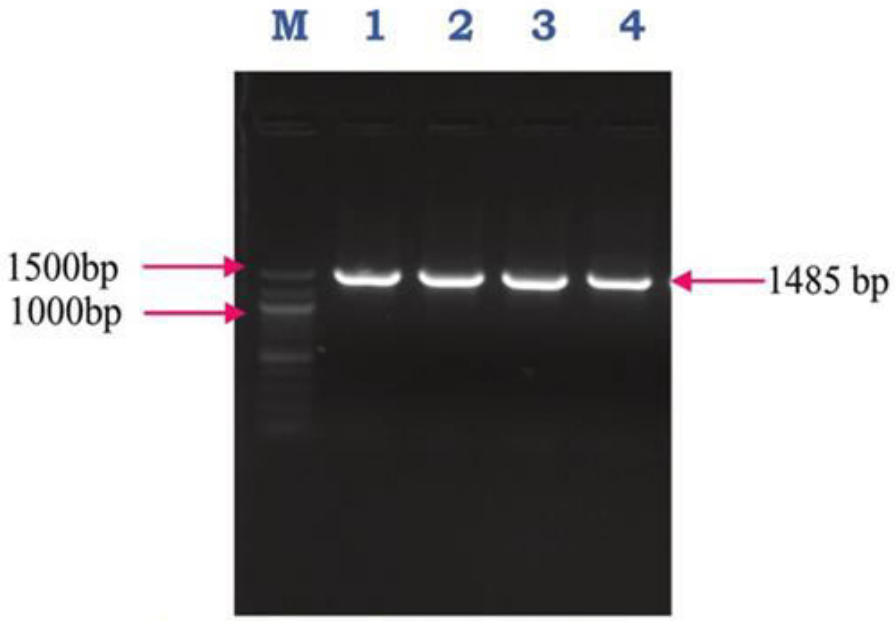


Fig. 1. PCR amplification with 16s r-DNA primers

There are two major clusters in the present dendrogram, one group representing the standard strains *P. fluorescens* and its closely affiliated strain JS16 while other group is represented by standard strains *P. plecogoccida*, closely affiliated strain and JS31 and JS7 and JS52 closely affiliated *P. putida*. Based on the above antagonistic and plant growth promoting tests, 4 isolates were subjected for 16S rRNA gene sequence identification. It was found that these isolates were highly homology to *P. fluorescens, P. plecoglossicida*

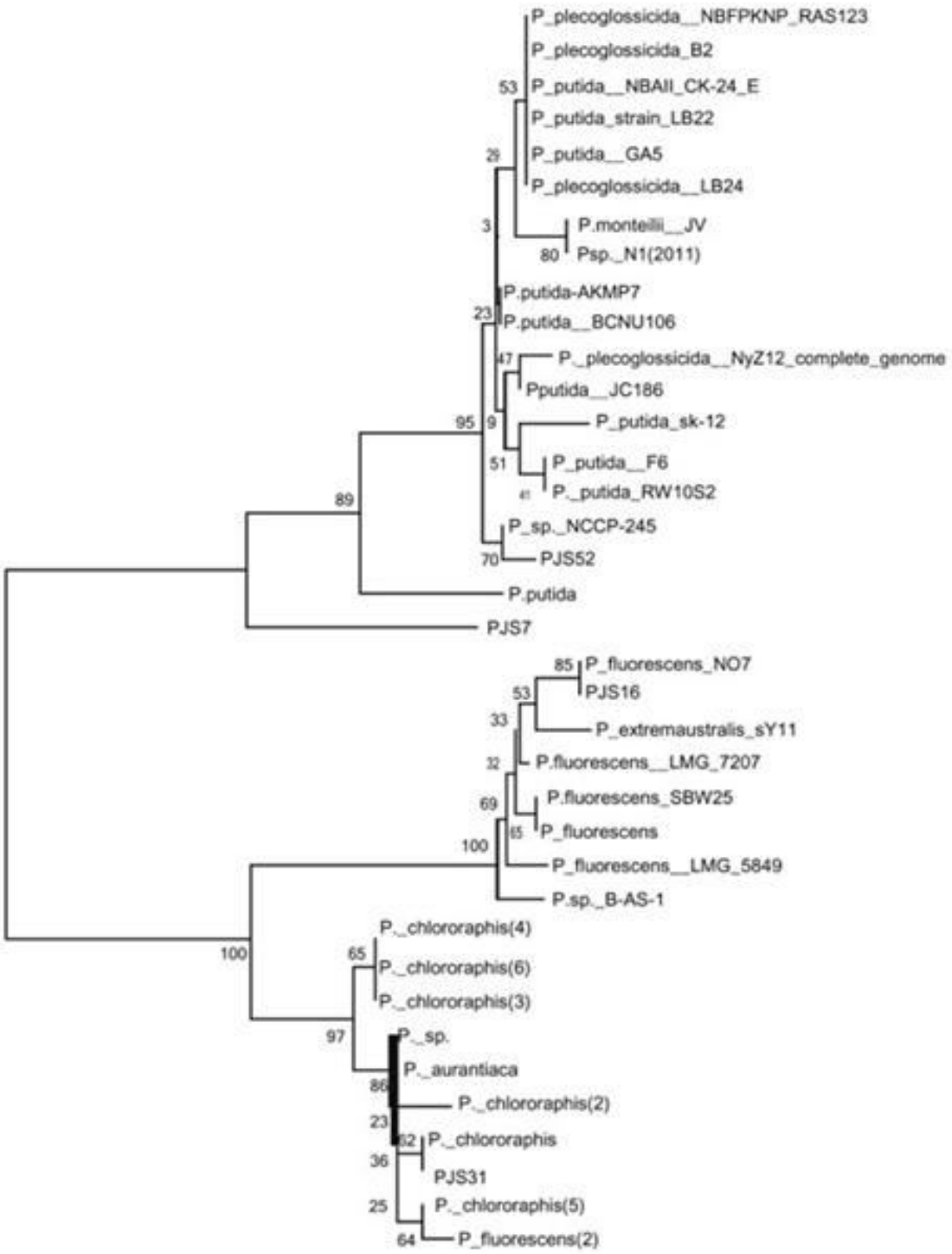


**Fig. 2. Neighbor joining dendrogram of Pseudomonas fluorescens isolates of groundnut. The number of each branch is the percentage of time the group of strains in that branch occurred, based on 1000 cycles in bootstrap analysis.**

## DISCUSSION

In recent years, much attention has been given to the antagonistic activities and bio-fertilizing abilities of fluorescent Pseudomonads from agricultural crop plants. Traditional morphological differences were difficult to distinguish these species differentiation by morphological, biochemical methods. Studies on the diversity of antagonistic rhizobacteria using molecular techniques are important not only for understanding their ecological role in the rhizosphere, but also for characterization of the biological control agent for registration, patenting, recognition and quality checking (Marten *et al*., 2000). The most extensive studies regarding the phylogenetics of *Pseudomonas* spp. have been based on the 16S rRNA gene (Kersters *et al*., 1996; Anzai *et al*., 2000; Porteous *et al*., 2002). The 16s rRNA, based sequence analysis identified four different species of fluorescent Pseudomonads (*P. fluorescens, P. putida,* and *P. plecoglossicida).* Clustering based on genetic data revealed three major groups such as *P. Fluorescens* group, *P. putida* group and *P. Plecoglossicida* group.

The use of the 16S rRNA gene as a phylogenetic marker has been criticized, as it assumes one molecule reflects the evolution of an organism and the rate of evolution of the 16S rRNA gene is not sufficient to permit resolution of intrageneric relationships (Yamamoto *et al*., 2000). Despite this, there remains widespread confidence in 16S rRNA based phylogenetic inferences (Vandamme *et al*., 1996; Ludwig & Schleifer, 1999).Although sequence analysis of 16s rRNA has been used for the delineation of bacterial species (Wayne *et al*., 1987), phylogenetic analyses resulted into better resolution. The lack of similarity between clusters from phenotypic and genetic data was interpreted as a result of high diversity among antagonistic fluorescent Pseudomonads. Results of present investigation indicated a high degree of functional diversity among antagonistic fluorescent Pseudomonads of groundnut rhizosphere.

## CONCLUSION

Knowledge on phenotypic and functional traits of antagonistic bacteria will help determine their fitness for successful bio-fertilization and biological control. The present study showed a high degree of functional and genotypic diversity among fluorescent pseudomonads in the groundnut rhizosphere. Amplification of 16S rRNA gene region is the suitable technique for *Pseudomonas* strain identification is more accurate. Therefore, data presented in the present investigation on genetic and functional characterization of antagonistic fluorescent Pseudomonads might be useful for designing biological control strategies through the identification of novel strains that are ecologically competent. This information helps us identify efficient strains with diverse mechanisms directly from the soil.

## REFERENCES

Anzai, Y., Kim, H., Park, J. Y., Wakabayashi, H. & Oyaizu, H. (2000). Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. Int J Syst Evol Microbiol 50, 1563–1589

Ausubel, F.M., F. Katagiri, M. Mindrinos and J. Glazebrook (1995) Use of *Arabidopsis thaliana* defense-related mutants to dissect the plant response to pathogens. Proc. Natl. Acad. Sci. USA92:4189-4196.

De Freitas, J. R., and J. J. Germida. 1991. Psuedonzonas cepacia and Psez~don~onas putida as winter wheat inoculants for biocontrol of Rhizoctania rolaizi. Can. J. Microbial. 37:780-784.

Higgins D, Bleasby, A., & Fuchs, R. (1992) ClustalV: Improved software for multiple sequence alignment. Comput. Applic. Biosci. 8, 189-191.

Keel, C. and Defago, G., 1997, Interactions between beneficial soil bacteria and root pathogens: mechanisms and ecological impact. In: *Multitrophic Interactions inTerrestrial System*, Eds. Gange, A.C., Brown, V.K., Oxford Blackwell Science, pp.27-47.

Kersters, K., Ludwig, W., Vancanneyt, M., Devos, P., Gillis, M. & Schleifer, K. H. (1996). Recent changes in the classification of pseudomonads: an overview. Syst Appl Microbiol 19, 465–477

Marten P, Smalla K, Berg G (2000). Genotypic and phenotypic differentiation of an antifungal biocontrol strain belonging to Bacillus subtilis. J. Appl. Microbiol. 89:463

O'Sullivan, D.J. and O'Gara, F., 1992, Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiology Review, 56: 662-676.

Palleroni, N. J. (1993). Pseudomonas classification. A new case history in the taxonomy of Gram-negative bacteria. Antonie Leeuwenhoek. Int J Microbiol 64, 231–251.

Porteous, L. A.; Widmer, F. and seidles, R. T. (2002). Multiple enzyme restriction fragment length polymorphism analysis for high resolution distinction Pseudomonas 16S r RNA genes. J. Microbiol. Meth. 51:733-843.

Sambrook and Russell J Sambrook, D.W Russell, Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2001).

Wayne, L. G., Brenner, D. J., Colwell, R. R. (1987). International Committee on Systematic Bacteriology. Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol. 37: 463-464.

Yamamoto, S., Kasai, H., Arnold, D. L., Jackson, R. W., Vivian, A. & Harayama, S. (2000). Phylogeny of the genus Pseudomonas: intrageneric structure reconstructed from the nucleotide sequences of gyrB and rpoD genes. Microbiology 146, 2385–2394.

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