

Isolation, Identification and Computational Studies of *Pseudomonas Putida* Evhs2 in Tannery Effluent

K. Tulasi Ramakrishnan*, C. Sivasubramanian
and R. Raja Kumar^δ

*Department of Environment and Herbal Science, Tamil University,
Thanjavur – 613 403, Tamil Nadu, India.*

*^δDepartment of Biotechnology, Marudupandiar College,
Thanjavur – 613 403, Tamil Nadu, India.
E-mail: *tulasi_bio@yahoo.com*

Abstract

A study about isolation, identification and analysis of bacteria in tannery effluent. Here the tannery effluent is used as a sample for the entire analysis. A bacterial strain, designated EVHS2 was isolated from the Tannery effluent sample collected from Sembattu, Trichirappalli district, Tamil Nadu, India. It is identified by a series of biochemical tests and the identification is confirmed by using molecular approach. On the basis of the bacterial 16S rRNA sequence, phylogeny and comparison of this gene sequence with the sequence in GENBANK database, it is considered that the isolate is closely related to the members of *Pseudomonas putida*. Phylogeny and molecular evolutionary analysis were conducted using BLAST (Basic Local Alignment Search Tool) tool. The sequence of 16S rRNA (*Pseudomonas putida* EVHS2) is submitted to GENBANK in NCBI database (accession number is JF719292.1. It showed 99% of sequence similarity. Separate chromatogram was constructed for both the primers (forward and backward) amplified, using FINCH TV software.

Keywords: *Pseudomonas putida* EVHS2, Phylogeny, Effluent, FINCH TV, 16S rRNA.

Introduction

Molecular biology methods are now being employed to study bioremediation since a comprehensive understanding of microbial ecology is required to gain maximum benefits from this process. A number of studies have reported the occurrence of conjugative gene transfer between bacteria in soil through appropriate plasmid-borne

catabolic genes into competitive indigenous bacterial populations during bioremediation of contaminants at the industrial sites. Therefore, we need to understand the role of catabolic genes by molecular cloning and characterization for degradation of a particular organic compound so that it can be applied for bioremediation of contaminated groundwater and soil.

The relatively new field of molecular microbial ecology which makes use of molecular biology techniques to study microbial ecology is increasing our understanding of microbial diversity as it has revealed the presence of viable but non-culturable bacteria. Molecular microbial ecology describes the microbial diversity based on DNA sequences without cultivation. DNA is extracted directly from environmental samples so that the cultivation bias is eliminated. The ability to monitor expression of specific catabolic genes may be of great importance in determining the feasibility of bioremediation *in situ*.

Pseudomonas putida is a good candidate for metabolic engineering and genetic manipulation applications for expression of genes encoding several degradative enzymes. Therefore, a *Pseudomonas putida* strain was engineered to increase the efficiency of degradation of naphthalene and salicylate (Samanta *et al.*, 2001). In this strain, utilization of glucose (a simple carbon source) was blocked through metabolic engineering making it primarily dependent on the more complex carbon source, i.e. the organic compound in question (naphthalene/ salicylate) thus degrading it more efficiently.

Taxonomically, *Pseudomonas sp* belongs to the rRNA group I of the gamma subclass of Proteobacteria (Palleroni, 1993; Kersters *et al.*, 1996). The species within this group have been differentiated on the basis of their DNA/Dna homologies (Johnson and Palleroni, 1989). This method of differentiating bacterial species has for several years been the most reliable, since traditional classification of bacteria based on phenotypic features does not always correlate with molecular taxonomy (Woese, 1987). Other methods based on cataloguing 16S rRNA have been shown to be reliable and effective (Woese *et al.*, 1984; Woese, 1987). Indeed, using the 16-rDNA approach, Woese *et al.* (1990) were able to show definitively that life can be divided into three major lines of descent presently named Archea, Bacteria and Eukarya.

Methodology

Isolation and Identification

The bacteria were identified based on colony characteristics, Gram staining method and by various biochemical tests as given by Bergey's (1984) Manual of Systematic Bacteriology.

Amplification

Bacterial isolates are sub cultured on Blood Agar Plate. A single colony was obtained from a fresh subculture and resuspended in master mixture. A 380-bp fragment of the 16S rRNA (universal primer) was used as the target. For 50µl reaction, 1µl of 200Mm dNTPs, 5µl of Reaction buffer (Tris with MgCl₂),

10PM Forward primer RW01 (FP) 5'-AACTGGAGGAAGGTGGGGAT-3'

10Pm Reverse primer DG74(RP) 5'-AGGAGGTGATCCAACCGCA-3'

1.2u/μl of Tag Polymerase were used, amplification was carried out in Thermal Cycler PTC 200(MJ Research), and a standard three-step protocol was used for all reactions, including 35 cycles for each reaction with an annealing temperature of 55°C and a 10-min extension time.

16S rRNA Sequencing

The amplified product was purified using Bio basic Inc, Bangalore Genei, India. Cyclic PCR was carried out using Big Dye Terminator Ver 3.1. Each purified template was sequenced on both strands, reaction was carried out using Genetic Analyzer 3130 (ABI System).

Computational Studies

The sequences obtained were then used to perform nucleotide-nucleotide searches utilizing the BLAST database at the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST/>). BLAST outputs were sorted based on maximum identity, and identifications were made. And obtained the homology by Neighbour Joining method using BLAST (Basic Local Alignment Search Tool) tool and evolutionary tree was constructed. The nucleotide chromatogram was constructed for both the primers which are amplified, using FINCH TV software.

Results and Discussion

The sequences obtained were matched with previously published sequences available in NCBI using BLAST (Altschul *et al.*, 1997).

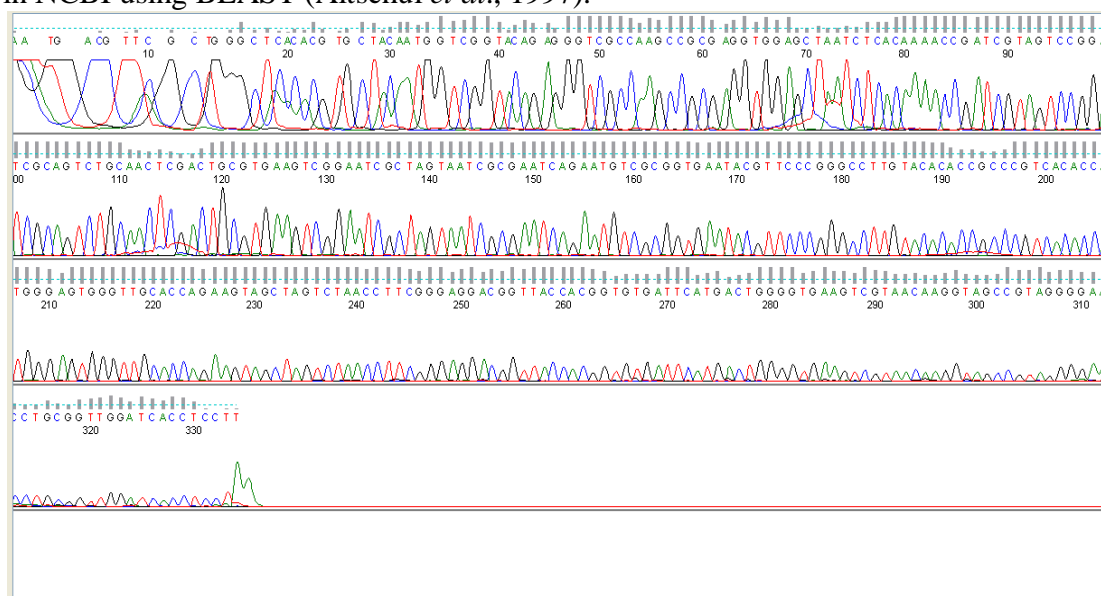


Figure 2: Chromatogram of 16S rRNA Sequence by Forward primer for *Pseudomonas putida* EVHS2 constructed by FINCH TV Software.

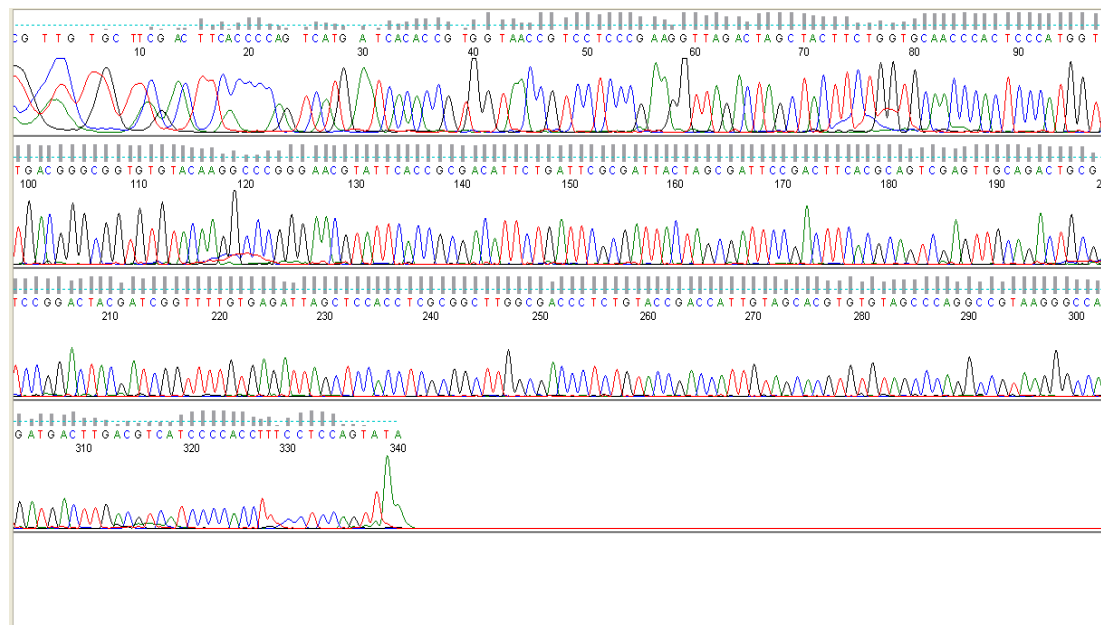


Figure 3: Chromatogram of 16S rRNA Sequence by Reverse primer for *Pseudomonas putida* EVHS2 constructed by FINCH TV Software.

BLAST searches yielded at least 85% & for closely related species >90% of query coverage. *Pseudomonas putida* strain CT363 is closely related species, they are very to identify with 16S rRNA gene sequence alone. The data are simultaneously made available to EMBL in Europe and DNA Data bank of Japan. Figure 2 and Figure 3 represents the Chromatogram of 16S rRNA Sequence amplified by both Forward and Reverse primer for *Pseudomonas putida* constructed by FINCH TV Software.

Knowledge of micro organisms in the environment in the past depends mainly on the study of pure culture in the laboratory. Rarely are microbes so captured. Studies of several types of environments estimate that more than 99% of micro organisms seen microscopically and are not cultivated by routine techniques. (Amann *et al.*,1995). With the sequence based taxonomic frame work of molecular trees, only a gene sequence, not a functioning cell, is required to identify the organisms in terms of its phylogenetic type. The occurrence of phylogenetic type organisms, “Phylotypes” and their distributions in the natural communities can be surveyed by sequencing rRNA genes obtained from DNA isolated directly from the organism. Analysis of microbial ecosystem in this way is more than a taxonomic exercise because the sequence provides the experimental tools. For instance, molecular hybridisation probes, which can be used to identify, monitor and study the microbial inhabitants of natural ecosystems (Hugenholtz and Pace, 1996).

The tree (Figure 1) can be considered a rough map of evolution of genetic core of the cellular lineages that led to the modern organisms (sequences) included in the tree. The tree of occurrence of evolutionary events cannot be extracted reliably from phylogenetic trees, despite common attempts to do so. Time cannot be accurately correlated with sequence change because the evolutionary clock is not constant in

different lineages. This disparity is due to the fact lines leading to the different reference organisms are not all the same length; these different lineages have experienced different extents of sequence change. Nevertheless, the order of occurrence of branching in the trees can be interpreted as a genealogy, and intriguing insights into the evolution of cells are emerging (Woese, 1987).

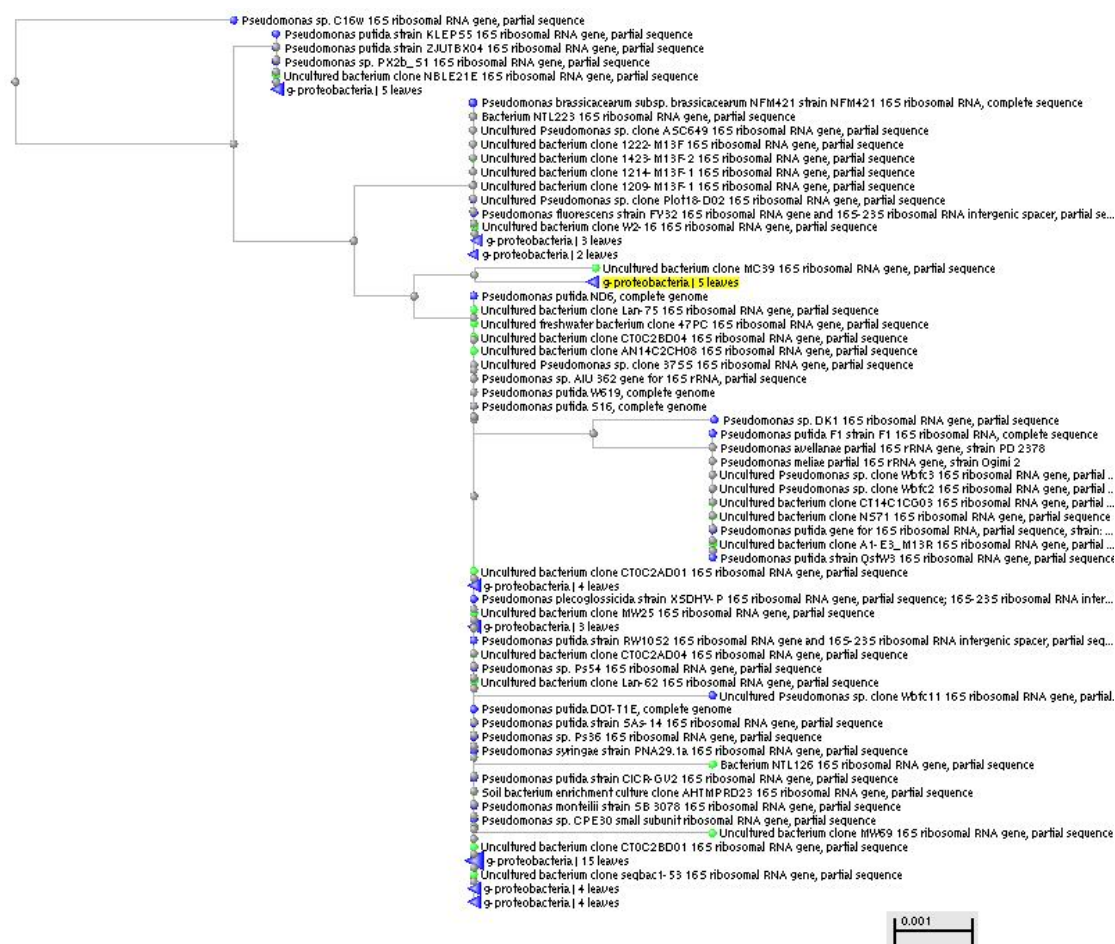


Figure 1: Phylogenetic Tree of *Pseudomonas putida* EVHS2 constructed by Neighbour Joining method using BLAST Program.

Conclusion

The sequence of 16S rRNA (*Pseudomonas putida* EVHS2) is submitted to GENBANK in NCBI database (Accession number is JF719292.1). BLAST searches yielded at least 85% & for closely related species >90% of query coverage. *Pseudomonas putida* strain CT363 is identified as closely related species.

References

- [1] Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman., (1990). "Basic local alignment search tool". J. Mol. Biol., 215:403–410.
- [2] Amann,R.I., Ludwig, W., and Schleife, K.H.(1995). "Phylogenetic identification and *insitu* detection of individual microbial cells without cultivation". Microbiol., 59:143-169.
- [3] Bergey's Manual., (1984). "Bergey's Manual of Systematic Bacteriology". Williams & Wilkins, Baltimore: USA.
- [4] Hugenholtz,P., and Pace, N.R.(1996). "A molecular view of microbial diversity and the biosphere". Trends Biotechnol., 14,:190.
- [5] Johnson, J.L. and Palleroni, NJ. (1989). "Deoxyribonucleic acid similarities among *Pseudomonas* species". International Journal of Systematic Bacteriology, 39: 230235.
- [6] Kersters, K; Ludwig, W; VanCanneyt, M; De Vos, P; Gillis, M and Schleifer, K-H.(1996). "Recent changes in the classification of the *Pseudomonas sp*: an overview". Systematic Applied Microbiology, 19: 465–477.
- [7] Palleroni, N.J. (1993). "*Pseudomonas* classification: A new case history in the taxonomy of Gram-negative bacteria". Antonie van Leeuwenhoek, 6231251.
- [8] Samanta, S. K., Bhushan, B. and Jain, R. K., (2001). "Efficiency of naphthalene and salicylate degradation by a recombinant *Pseudomonas putida* mutant strain defective in glucose metabolism". Appl.Microbiol. Biotechnol.,**55**, 627–631.
- [9] Woese, C.R, ; Kandler, O. and Wheelis, M.L. (1990). "Towards a natural system of organisms: Proposal for the domains Archea, bacteria and Eucarya". Proceeding Natural Academy of Science, 7, 4578–4579.
- [10] Woese, C.R. (1987). "Bacterial evolution". Microbial Review, 6, 60–69.
- [11] Woese, C.R; Blanz, P. and Hahn, C.M., (1984). "What isn't *Pseudomonas sp*: the importance of nomenclature in bacterial classification". Systematic Applied Microbiology.**5**, 179–195.