International Journal for Life Sciences and Educational Research
Vol. 3(2), pp. 31-35, April - 2015
Available online at http://www.ijlser.com
E-ISSN : 2321-1229; P-ISSN : 2321-1180

Research Article

Genetic diversity of phosphate solubilizing pseudomonads isolated from rice plant (*Oryza sativa* L.) rhizospheric soil, Sivagangai, India

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Article History : Received 16 March 2015, Accepted 24 April 2015

Abstract

Genetic diversity of plant growth-promoting rhizobacteria (PGPR) associated with rice plant (*Oryza sativa* L.) in rhizospheric soil collected from the rice fields in Sivagangai, Tamilnadu has been analyzed in the present study. A total of 13 strains among 961 isolates were randomly selected for studies based on the genus specific confirmation by the *Pseudomonas* specific primer. Phylogenetic analysis based on 16S rDNA gene sequences generated 3 clusters showing more than 90% of boot strap value.

Keywords : Pseudomonas, Plant Growth Promotion, PSB, 16S rDNA and Rhizobacteria

Introduction

Paucity of phosphorous in the soil is a major limitation to crop production. Rice crop land, soil possesses a considerable amount of phosphorous due to the regular application of chemical fertilizers. Plants absorb only inorganic form of phosphorous. The level of inorganic phosphorus is low in the soil as most of the phosphorous is present as insoluble form. Besides, a large proportion of chemical fertilizers added to the soil are also converted into insoluble form and become unavailable to the plants (Rodriguez and Fraga, 1999). However, the increasing use of chemical inputs cause negative effects, i.e., development of pathogen resistance and non-target environmental impacts (De Weger *et al.,* 1995). The ruthless application of chemical fertilizers has led to the decline in the population of beneficial microbes that include PGPRs.

Typically, *Pseudomonas* is a rod shaped, gram-negative, one or more polar flagella, motile, aerobic, non-endospore forming bacteria. *Pseudomonads* belong to the family gamma Proteobacteria. The genus *Pseudomonas* is one of the most diverse genera, and its taxonomy has undergone many changes since earlier descriptions (Palleroni, 1984). Sequencing of the 16S rDNA gene has redistributed some of the former *Pseudomonas* species into other genera, in particular, into the alpha, beta or gamma subclasses of Proteobacteria (Kersters *et al.,* 1996).

The members of the genus *Pseudomonas* (sensu stricto) belong to Palleroni's RNA group I, in the Gamma-Proteobacteria. *Pseudomonas* is a diverse group with more than 60 species exhibiting varied lifestyles in a wide range of environments, including soil, water, plant surfaces, and animals. Furthermore, *Pseudomonads* are known for their ubiquity in the environment, utilization of a striking variety of organic compounds as energy sources (Wu *et al.,* 2010). The remarkable ecological and metabolic diversity of *Pseudomonas* spp. is reflected in the genomes of these bacteria.

Cosmopolitan distribution of *Pseudomonads* is further ascertained by its remarkable

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physiological and genetic adaptability. Genomic diversity is particularly apparent from the relatively small size of the core genome that is shared among *Pseudomonas* species (Gross and Loper, 2007). Further, the genomes of *Pseudomonas* spp., like those of many other bacteria, display a highly mosaic structure, being composed of relatively stable core regions interspersed with regions that vary among the strains (Silby *et al.,* 2011).

High degree of conservation of the 16S rDNA gene led to a small number of informative sites in its sequence (Anzai *et al.,* 2000). To date, the sequenced strains represent only a fraction of the diversity within the *Pseudomonads*, and much of the group's metabolic, ecological, and genetic diversity remains unexplored (Santos and Ochman, 2004). In the present study, *Pseudomonads* with plant growth promoting traits were isolated and phylogenetic relationship among the isolated was established based on the 16S rRNA gene.

Materials and Methods

Isolation

Soil samples were collected from rice field rhizospheric soil, Sivagangai district, Tamilnadu, India. Isolation of fluorescent *Pseudomonads* bacteria from rhizospheric soil was performed as described earlier (Sunish Kumar *et al.,* 2005). Briefly, soil suspension was obtained by shaking 10 g of soil sample in 90 ml of 0.1 M $MgSO_4.7H_2O$ buffer for 10 min at 180 rpm on a rotary shaker. Resulting suspensions were serially diluted and 0.1 ml aliquots of each dilution were spread on to King's medium B (KB) agar in triplicates. Purified single colonies were further streaked on to KB agar plates to obtain pure cultures. Stock cultures were made in Luria Bertani (LB) broth containing 50% (w/v) glycerol and stored at -86°C.

16S rRNA gene amplification, sequencing and phylogenetic tree analysis

Amplification of 16S rRNA gene was performed from the genomic DNA of strains using universal primers fD1 (5'-GAGTTTGATCCTGGCT CA -3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg *et al.,* 1991). PCR cocktails (50 µl) contained 50 pM of primer, 50 ng of genomic DNA, 1×Taq DNA polymerase buffer, 1 U of Taq DNA polymerase (Promega, Madison, WI, USA), 0.2 mM of each dNTP, and 1.5 mM MgCl2. Amplification was performed in a DNA thermo cycler (2400 cycler, Perkin Elmer International, Rotkreuz, Switzerland) at 94°C for 3 min, followed by 30 cycles of 10 s at 94°C, 1 min at 56°C and 30 s at 72°C with an extension of 72°C for 5 min. A 5 µl aliquot of each amplification product was electrophoresed on a 0.7% agarose gel in 1× TAE buffer at 50 V for 45 min, stained with ethidium bromide and the PCR products were visualized with a UV transilluminator. PCR (Explain) products were purified using Quick PCR purification column (Promega, Madison, USA). Purified PCR products were sequenced with automated DNA sequencer with specific primers using the facility at Macrogen Inc. (Seoul, Korea). To perform molecular phylogenetic analyses, reference sequences required for comparison were downloaded from the EMBL database using the site http://www.ncbi.nlm.nih.gov /Genbank website. All the sequences of 16S rRNA were aligned using the multiple sequence alignment program CLUSTAL W (Larkin *et al.,* 2007).

The aligned sequences were then checked for gaps manually, arranged in a block of 600 bp in each row and saved as molecular evolutionary genetics analysis (MEGA) format in MEGA version 5.0. To obtain the confidence values, the original data set was resampled 1000 times using the bootstrap analysis method. The bootstrapped data set was used directly for constructing the phylogenetic tree using the MEGA v5.0 program for calculating the multiple distance matrixes and analysis (Tamura *et al.,* 2011; Kumar *et al.,* 2012). The multiple distance matrix obtained was then

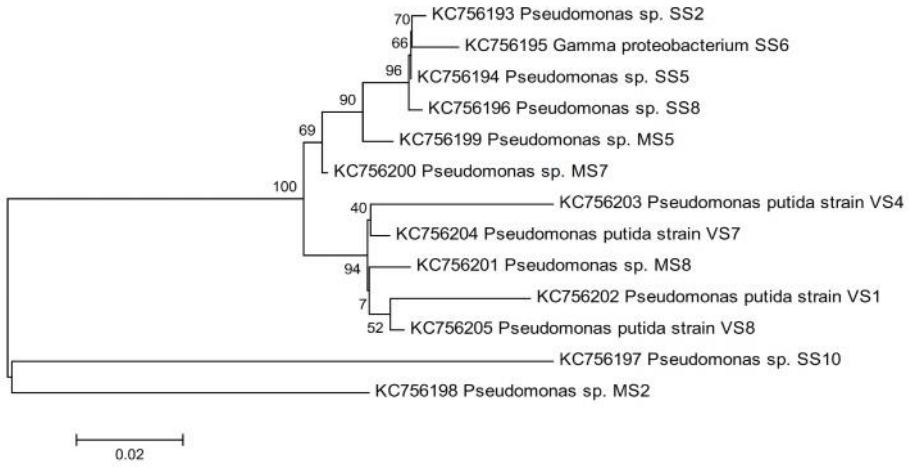

Fig.- 1. Evolutionary relationship of taxa using Neighbor-Joining method.

The analysis involved 13 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 354 positions in the final dataset.
Evolutionary analyses were conducted in MEGA5.

used to construct phylogenetic trees using neighbor-joining (NJ) method (Saitou and Nei, 1987).

Results and Discussion

The nucleotide sequences of 16S rRNA were deposited in GenBank. The accession numbers of the 16S rRNA nucleotide sequences of the strains were KC756193 *Pseudomonas* sp. SS2; KC756194 *Pseudomonas* sp. SS5; KC756195 Gamma proteobacterium SS6; KC756196 *Pseudomonas* sp. SS8; KC756197 *Pseudomonas* sp. SS10; KC756198 *Pseudomonas* sp. MS2; KC756199 *Pseudomonas* sp. MS5; KC756200 *Pseudomonas* sp. MS7; KC756201 *Pseudomonas* sp. MS8; KC756202 *Pseudomonas* putida strain VS1; KC756203 *Pseudomonas* putida strain VS4; KC756204 *Pseudomonas* putida strain VS7; KC756205 *Pseudomonas* putida strain VS8.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.35727517 is shown (Fig.-1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

There have been many attempts to establish clear species boundaries, or 'cut-offs', between bacterial species, based on bioinformatics analysis of comparisons of whole genomes or phylogenetic similarities between strains. The recognized 70% genomic DNA–DNA similarity by direct hybridization is the experimental level that is accepted for species circumscriptions in bacterial taxonomy. Conserved DNA measured with blast in the 16 sequenced genomes determines a species boundary of approximately 80%. This corresponds to a phylogenetic distance of 97% in the multi-genic analysis.

According to Spiers and colleagues (2000), extraordinary phenotypic and genetic diversity within Pseudomonas showed no definite pattern of distribution that could precisely define any of the lineages. 16S rDNA was included because, as a universal marker, it permitted the ascription of a strain to the genus and allowed comparisons

between very divergent bacteria (Santos and Ochman, 2004). Neighbor-joining method was selected as the tree-building method. This method is routinely used in Pseudomonas taxonomic studies, and its accuracy has been widely demonstrated (Mulet *et al.,* 2010). Thus, the use of a multigenic approach to give an actual view of the phylogenetic interrelationships of such a complex genus has become inevitable. This need becomes apparent because of the high number of new *Pseudomonas* species described each year.

Acknowledgements

We sincerely thank the Principal RIE, Mysore, Prof. Geetha G Nair and Prof. G.V. Gopal Botany Section, DESM, RIE, Mysore, authorities of the National Council of Educational Research and Training, New Delhi, and the volunteers for providing us the support to bring out this paper.

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