



## Rapid Methods for Identification of Plant-Growth-Promoting Rhizobacteria

Sangita Sahni<sup>1</sup> and Bishun Deo Prasad<sup>2\*</sup>

<sup>1</sup>Department of Plant Pathology, TCA, Dholi, Dr. Rajendra Prasad Central Agricultural University, Pusa, Bihar, India.

<sup>2</sup>Department of Molecular Biology and Genetic Engineering, Bihar Agricultural College, Sabour, BAU, Sabour, Bhagalpur, Bihar, India.

### Authors' contributions

This work was carried out in collaboration among all authors. Authors SS and BDP designed the study, managed the literature searches, wrote the protocol and wrote the manuscript. All authors read and approved the final manuscript.

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### ABSTRACT

The growing human population has put enormous pressure on agriculture to increase production and productivity, which has resulted in the widespread usage of agrochemicals. The indiscriminate use of agrochemicals has harmed soil fertility and resulted in significant environmental contamination, impacting human health. The discovery and implementation of bacterial isolates with Plant Growth Promoting Rhizobacteria (PGPR) have enormous potential for reducing the usage of chemical fertilizers, insecticides, and herbicides. However, the identification of bacterial isolates is a prerequisite to utilize them for agricultural purposes. Traditional methods involve culturing microbes using a range of nonselective and selective enrichment methods, followed by biochemical confirmation among others. Traditional methods involve culturing microbes, followed by morphological biochemical confirmation etc. In the present investigation, we describe a fast and effective approach for isolating and identifying bacterial isolates, followed by phylogenetic analysis and submission to GenBank.

**Keywords:** Plant Growth Promoting Rhizobacteria (PGPR); 16S rDNA; Gram staining, Phylogenetic analysis; GenBank.

\*Corresponding author: E-mail: [bdprasadbau@gmail.com](mailto:bdprasadbau@gmail.com);

## 1. INTRODUCTION

The increasing human population has put tremendous pressure on agriculture to enhance its production and productivity [1,2]. The pressure to increase production and productivity abruptly has caused excessive use of agrochemicals [3]. The indiscriminate use of agrochemicals has deteriorated soil fertility and cause huge environmental pollution leading to human health [4]. The unjudicial use of chemical fertilizers due to lack of information or due to subsidiary scheme has further deteriorated the soil health [5,6]. The excessive use of nitrogen fertilizers has led to the production of nitrous oxide (N<sub>2</sub>O), which causes global warming [7]. Although the immediate advantage appeared due to excessive use of various agrochemicals and fertilizers, their long term use magnifies environmental and public health threats. However, farming without the use of these chemicals not only reduces the yield drastically but also reduces soil fertility. Numerous reports suggest the use of organic fertilizers, which could reduce the use of chemical fertilizers [8,6]. However, the effectiveness of organic fertilizers doesn't comparable to that of chemical fertilizers. Several reports advocates the use beneficial rhizobacteria (plant growth promoting rhizobacteria), which has potential to reduce the dependency of agro-chemicals significantly or completely if amended with organic fertilizers [9]. The use of PGPR as inoculants provides an environmentally sustainable approach to increase stress tolerance, management of diseases and other traits leading to enhance productivity [10,11]. The potentiality of PGPR in agriculture is steadily increased as it offers an attractive way to replace the use of chemical fertilizers, pesticides and other supplements [12]. Numerous PGPR isolates have been identified and tested in field and laboratory conditions [10] however, the effectiveness of PGPR strains depends upon several factors including plant genotypes, abiotic and biotic stresses [13,14]. Therefore, the precise identification and screening of PGPR isolates are prerequisite to utilize them for agricultural purposes. In the present investigation, an attempt was made to provide a simple and rapid identification of rhizobacteria in the laboratory.

## 2. MATERIALS AND METHODS

### 2.1 Isolation of Bacterial Isolates

In present investigation, serial dilution method was used for isolation of bacterial isolates, as

described previously [15,16]. In brief, 10g of soil (usually rhizospheric soil) sample is suspended in 90 ml of sterile distilled water, mixed well by swirling. Transfer 1 ml of soil suspension into 9 ml sterile distilled water. Similarly, a series of dilutions (10<sup>-7</sup>) were prepared under aseptic condition. Bacteria were isolated by plating 100 µl of soil suspension on nutrient agar plate and incubate it at 30 °C for 12-24 h. The colonies appeared on petriplates were visually characterized on the basis of colour, shape, size, elevation etc. Colonies exhibiting prolific growth were selected and streaked for fresh nutrient agar plates.

### 2.2 Gram Staining

Gram staining is very useful method for identifying bacteria and classifying them into two major groups: the Gram-positive and Gram-negative. The purified colonies were inoculated in 3 ml of nutrient broth grown till 0.6 OD. For gram staining, a smear/thin film is made on a clean microscopic glass slide, air dry and heat-fix the smear by passing through flame. The fixed bacterial smears were stained with crystal violet as a primary stain for 1 minute and rinse the slide with water. Then, stained the slide using iodine solution as a mordant for 1 minute and rinsed with water. The slide was then decolorized rapidly with alcohol and rinsed with water. After this the slide was then counter stained with Safranin for 1 minute and rinsed with water and examined under the microscope.

### 2.3 Bacterial DNA Isolation

Genomic DNA of the bacterial isolate was extracted using CTAB protocol with slight modifications [17]. In brief, 5 mL of freshly grown bacterial culture in suitable centrifuge tube was centrifuged at 10,000 rpm for 1 min. Supernatant was discarded and resuspended the bacterial pelleted in 750 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Twenty micro litre of lysozyme (100 mg/ml) was added, mixed well by inversion and incubated at 37 °C for 10 min. then, 40 µl of 10% SDS and 8 µl of proteinase K (10mg/ml) were added to the lysate, mixed well and incubated for 30 min at 56 °C. One hundred micro litre of 5 M NaCl was added, mix well and then 100 µl of preheated (65 °C) CTAB/NaCl (4.1 g NaCl was dissolved in 80 ml of water and slowly 10 g CTAB was added while heating about 65°C and stirring. The final volume was adjusted to 100 ml of nucleases free water and sterilize by autoclaving and incubated at 65 °C

for 10 min. After incubation, equal volume of chloroform: isoamyl alcohol (1:1) solution was added to the bacterial lysate, mixed by inversion and centrifuged at 1000 rpm for 5 min. resulting aqueous solution was transferred to a clean eppendorf or micro centrifuge tube. DNA was precipitate by added 0.6 volume of isopropanol, mixed well by inversion, incubated on ice for 10-15 min, and centrifuged at 10,000 rpm for 10 min. Supernatant was discarded and DNA pellet was washed with 70% ethanol. Finally, DNA pellet was resuspended in 100 µl of nucleases free water, checked on agarose gel and used for PCR analysis.

## 2.4 PCR Amplification and Sequencing

PCR amplification of the 16S rRNA gene fragment was done by using 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-TACGGTTACCTTGTACGACTT-3) primers. The reaction mixtures composed of 5 µl 10xPCR buffer, 0.2 µM dNTPs, 1 µM each primer, 5 µl DNA template, 1 units Phusion® High-Fidelity DNA Polymerase (NEB) and sterile deionized water to a final volume of 50 µl. PCR profile was kept as follows: initial denaturation at 94°C for 3 min, followed by 25 cycles of denaturation (94°C, 30 sec), annealing (51°C, 30 sec), extension (72°C, 1 min), and final extension at 72°C for 5 min. The amplified PCR products were agarose-gel-eluted and purified using. The purified gel-eluted rDNA fragments were sequenced with primers: 27F and 1492R.

## 2.5 Identification of Isolates by 16S rRNA Sequence Analysis

The sequence data obtained from forward and reverse primer were analysed using Bioedit version 7.2.5 [18]. Consensus sequences were obtained by performing pairwise alignment of sequences and used for identification of bacterial isolate. The nucleotide sequences were searched against NCBI non redundant database (Nr) using BLAST (BlastN).The top 10 hits were used to construct a phylogenetic tree. The sequence was submitted to the 16S rRNA submission portal at NCBI (<https://submit.ncbi.nlm.nih.gov/>) to obtain accession number.

## 3. RESULTS AND DISCUSSION

### 3.1 Isolation of Bacteria from the Soil Samples

Rhizospheric soil samples were collected from research farm of TCA, Dholi. The collected soil

samples were used for isolation of bacterial isolates using serial dilution method. A plethora of reports supports the serial dilution method for identification of useful bacteria [15]. In present investigation, two bacterial isolates (SS1 and SS2) were isolated and used for further characterizations.

### 3.2 Gram Staining

The isolates were preliminary characterized based on gram staining and colony morphology. The Gram stain is the common, important, and most used differential staining method for the phenotypic characterization of bacteria [19,20]. In present investigation gram staining were performed to characterize both the isolates. The results of gram staining were summarized in Table 1.

**Table 1. Gram staining of bacterial isolates**

Bacterial isolates	Gram staining	Shape of bacteria
SS1	Gram-positive	rod-shaped
SS2	Gram-negative	rod-shaped

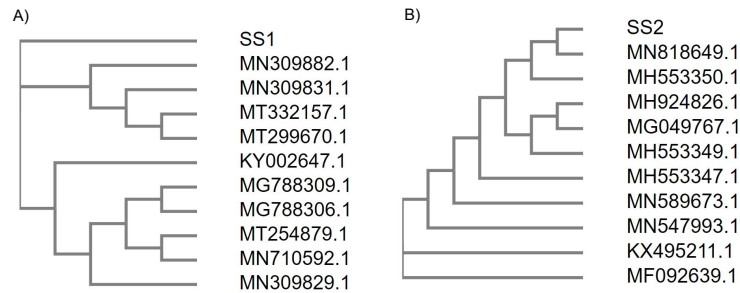
### 3.3 Identification of Isolates by 16S rDNA Sequence Analysis

Rapid identification of bacterial isolates were crucial for appropriate use in agricultural purposes. 16S rRNA sequencing has provided a powerful strategy for bacterial identification [21,22,23,20]. 16s rDNA was amplified from the genomic DNA of both the identified isolates. The amplified PCR product was gel purified and sequenced using both forward and reverse primer as mentioned in materials and methods. The conserved sequences were obtained using BioEdit software. Then, the bacterial strain sequences were compared to sequences that have been deposited in publically accessible databases (NCBI) using BLASTN. Top ten hits were used to create phylogenetic tree using Clustal Omega

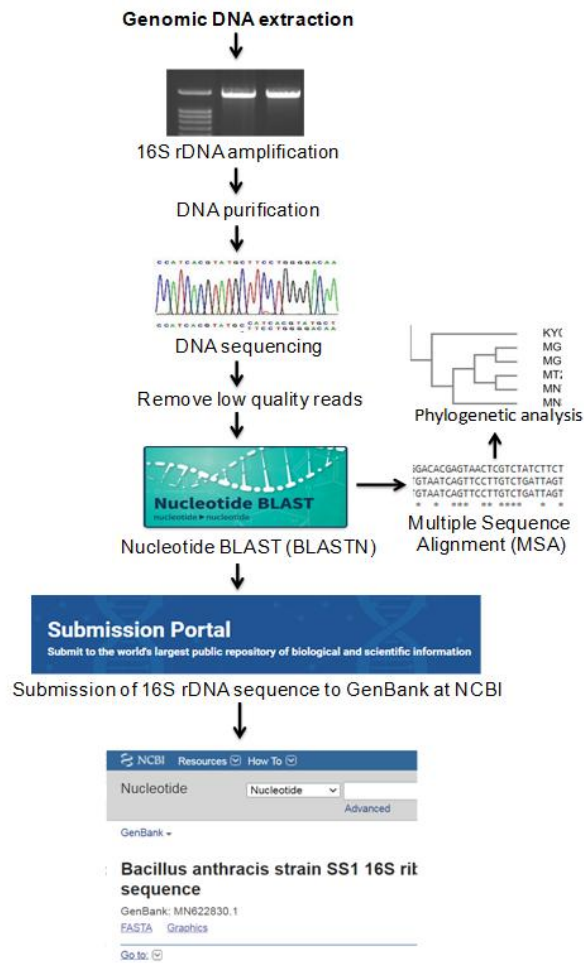
(<https://www.ebi.ac.uk/Tools/msa/clustalo/>) with default parameters (Fig. 1). BLASTN followed by analysis identified SS1 and SS2 bacterial isolates as *Bacillus anthracis* and *Providencia vermicola*, respectively. Sequencing of 16S rRNA gene is widely regarded as the backbone of bacterial phylogeny and taxonomic studies [24]. Clustal Omega [25] is a freely available open access web tool, used in multiple sequence analysis and phylogenetic analysis of 16s rDNA sequences ([26].

The 16S rDNA sequences of both the isolates were submitted to the GenBank at NCBI. The NCBI GenBank accession number for SS1 and SS2 are MN622830.1 and MN622828.1. GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>)

is a large collection of freely available nucleotide sequences including 16S rDNA [27]. A workflow for rapid identification of bacterial isolates has been summarized in Fig. 2.



**Fig. 1. Phylogenetic analysis of bacterial isolates based on 16S rDNA. A) Bacterial isolate showing SS1 close homology with *Bacillus sp.* B) Bacterial isolate showing SS2 close homology with *Providencia sp.***



**Fig. 2. Overview of identification of bacterial isolates using 16S rDNA analysis**

## CONCLUSION

Sequencing of 16S rDNA has provided a powerful strategy for bacterial identification. In present investigation, we have shown the stepwise procedure for bacterial isolation, gram staining, amplification and sequencing of 16S rDNA, phylogenetic tree construction and submission of 16S rDNA sequences to GenBank to obtain accession number. In summary, by following this procedure researchers can rapidly identify and utilize the potentials of bacterial isolates.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

- Josephson AL, Ricker-Gilbert J, Florax RJGM. How does population density influence agricultural intensification and productivity? Evidence from Ethiopia. *Food Policy*. 2014;48:142-152.
- Fróna D, Szenderák J, Harangi-Rákos M. The Challenge of Feeding the World. *Sustainability*. 2019;11:5816.
- Aktar MW, Dwaipayan SD, and Chowdhury A. Impact of pesticides use in agriculture: their benefits and hazards. *Interdiscip Toxicol*. 200;2(1):1–12.
- Wang MC, Gong M, Zang H-B, Hua XM, Yao J, Pang YJ, Yang YH. Effect of Methamidophos and Urea Application on Microbial Communities in Soils as Determined by Microbial Biomass and Community Level Physiological Profiles. *J. Environ. Sci. Health B*. 2006;41:399–413.
- Mahmood I, Imadi SR, Shazadi K, Gul A, Hakeem KR. Effects of Pesticides on Environment. In: Hakeem K., Akhtar M., Abdullah S. (eds) *Plant, Soil and Microbes*. Springer, Cham; 2016.
- Ye L, Zhao X, Bao E, Li J, Zou Z, Cao K. Bio-organic fertilizer with reduced rates of chemical fertilization improves soil fertility and enhances tomato yield and quality. *Sci Rep*. 2020; 10(1):177.
- Kusin FM, Akhir NIM, Mohamat-Yusuff F, Awang M. The impact of nitrogen fertilizer use on greenhouse gas emissions in an oil palm plantation associated with land use change. *Atmósfera*. 2015;28 (4):243-250.
- Oliveira AB, Moura CFH, Gomes-Filho E, Marco CA, Urban L, Miranda MRA. The Impact of Organic Farming on Quality of Tomatoes Is Associated to Increased Oxidative Stress during Fruit Development. *PLoS One*. 2013;8(2):e56354.
- Padmini OS, Wuryani S, Aryani R. Application of Organic Fertilizer and *Plant Growth-Promoting Rhizobacteria* (PGPR) to Increase Rice Yield and Quality. In: Taufik T. et al. (eds) *ICoSI 2014*. Springer, Singapore; 2017.
- Kumar S, Sahni S, Kumar B, Prasad SS. Multifarious Antagonistic Potentials of Native Pseudomonad Isolates from Rhizosphere as Biocontrol Agents for the Management of Chickpea Wilt. *Current Journal of Applied Science and Technology*. 2018;31(3):1-13.
- Leontidou K, Genitsaris S, Papadopoulou, A. et al. Plant growth promoting rhizobacteria isolated from halophytes and drought-tolerant plants: genomic characterisation and exploration of phyto-beneficial traits. *Sci Rep*. 2020;10:14857.
- Bhattacharyya PN, Jha DK. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J Microbiol Biotechnol*. 2012;28(4):1327-50.
- Martinez-Viveros O, Jorquera MA, Crowley DE, Gajardo G, Mora ML. Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *J Soil Sci Plant Nutr*. 2010;10:293-319.
- Beneduzi A, Ambrosini A, Passaglia, LM. Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. *Genetics and Molecular Biology*. 2012; 35(4):1044-1051.
- Kannan MK, Sethi S, Badoni A, Chamoli V, Bahuguna NC. Isolation and characterization of bacterial isolates from agriculture field soil of Roorkee region. *Journal of Pharmacognosy and Phytochemistry*. 2018;SP5:108-110.
- Somasegaran P, Hoben HJ. *Handbook for Rhizobia. Methods in Legume–Rhizobium Technology*. Heidelberg, NY: Springer; 1994.
- Wilson K. Preparation of genomic DNA from bacteria. *Curr Protoc Mol Biol*; 2001.
- Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Window 95/98/NT. *Nucleic Acids Symposium Series*. 1999;41:95–98.
- Mudili J. *Introductory Practical Microbiology*. Oxford: Alpha Science International Ltd; 2007.
- Nasfi Z, Busch H, Kehraus S, et al. Soil Bacteria Isolated From Tunisian Arid Areas

- Show Promising Antimicrobial Activities Against Gram-Negatives. *Front Microbiol.* 2018;9:2742.
21. Mehnaz S, Mirza MS, Haurat J, Bally R, Normand P, Bano A, Malik KA. Isolation and 16S rRNA sequence analysis of the beneficial bacteria from the rhizosphere of rice. *Can J Microbiol.* 2001;47(2):110-7.
  22. Ayyaz K, Zaheer A, Rasul G, Mirza MS. Isolation and identification by 16S rRNA sequence analysis of plant growth-promoting azospirilla from the rhizosphere of wheat. *Brazilian Journal of Microbiology.* 2016;47(3):542-550.
  23. Kai S, Matsuo Y, Nakagawa S, Kryukov K, Matsukawa S, Tanaka H, Iwai T, Imanishi T, Hirota K. Rapid bacterial identification by direct PCR amplification of 16S rRNA genes using the MinION™ nanopore sequencer. *FEBS Open Bio.* 2019;9:548–557.
  24. Lapage SP, Sneath PHA, Lessel EF, Skerman VBD, Seeliger HPR, Clark WA. *International Code of Nomenclature of Bacteria: Bacteriological Code, 1990 Revision.* ASM Press, Washington (DC); 1992.
  25. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994;22(22):4673-80.
  26. Ibal JC, Pham HQ, Park CE, Shin J-H. Information about variations in multiple copies of bacterial 16S rRNA genes may aid in species identification. *PLoS One* 2019;14(2):e0212090.
  27. Sayers EW, Cavanaugh M, Clark K, Ostell J, Pruitt KD and Karsch-Mizrachi I. GenBank. *Nucleic Acids Res.* 2020;48(D1):D84–D86.

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