

ISOLATION AND MOLECULAR IDENTIFICATION OF RHIZOBACTER FROM ROOTS OF *Salvadora persica* L. COLLECTED FROM HARAPPA, PUNJAB, PAKISTAN

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Received: December 19, 2025; Accepted: January 27, 2026

ABSTRACT

Salvadora persica L., is a perennial specie of family salvadoraceae renowned for its ethnobotanical uses due to its polypharmacological properties, and it is endangered due to its over exploitation. The aim of this study is the isolation and molecular identification of Rhizobacter in symbiotic association with *S. persica* L. acclimatised in the Harappa region of Punjab, Pakistan. Aseptically processed Rhizospheric soil samples were used to isolate bacteria by serial dilutions for plating on Luria Bertani agar medium. After proliferation and isolation of Gram positive *Bacilli*, the genomic DNA was extracted and brought under the 16S rRNA PCR. The isolate was identified as *Priestia megaterium* after conducting sequencing and BLAST analysis of 898bp amplicon with a satisfactory result of a 100% similarity. The acquired sequence was then deposited in NCBI GenBank under the Accession No. PQ136465 confirming its Phylogenetic taxonomic placement within the *Priestia* genus. The industrial uses of *P. megaterium* are recognized for plant nutrient solubilisation and phytohormone production promoting plant growth categorising this rhizobacterium as a promising bacterial isolate for biofertilizer research. This study accentuates the ecological importance and potential of native soil biomes and bacterial communities in symbiotic associations with the plants enhancing and conserving soil fertility creating resilience against climate change.

Keywords: *Priestia megaterium*, Climate change, Rhizobacteria, 16S rRNA, Biofertilizer, Symbiosis

INTRODUCTION

Salvadora persica L., widely deployed ethnobotanically for oral hygiene containing various phytometabolites notably flavonoids, terpenes, glycosides alkaloids, as well as minute traces of calcium, fluoride and sulphur compounds (Alam *et al.*, 2022). Its use in oral care has been validated by analytical standardizations (Farak *et al.*, 2021). Its ethnobotanical applications aside, *S. persica* L. has also been deployed in land reclamation efforts and agroforest developments (Sujata *et al.*, 2015). Although *S. persica* L. is native to the semi-arid thorn forests habitats of Pakistan (Varma *et al.*, 2018), it is under threat of extirpation from Pakistan due to constant harvesting for industrial use, urbanization and agricultural endeavours. Its susceptibility to multifaceted threats necessitates urgent *in situ* habitat conservation and *ex situ* conservation strategies (Umbreen *et al.*, 2025).

Just as any plant *S. persica* L. is inherently dependent on the intricate soil microbiome in a delicate equilibrium creating a rhizosphere, where roots are in direct contact with soil entertaining a diverse community of microorganisms crucial for plant life (Gupta, 2024). Within these rhizospheric community of microorganism, Plant Growth Promoting Rhizobacteria (PGPR) play a critical role performing nitrogen fixation, accelerating nutrient acquisition as well as producing phytohormones such as auxins. These rhizobacteria promote plant growth, making phosphate accessible to plants by solubilization, instigate resistance against pathogens and producing siderophores (Etesami and Adl, 2020; Teemofeeva *et al.*, 2023). Employing PGPR is an exemplary technique in terms of achieving sustainable agriculture, providing an ecologically apt alternative to chemical fertilizers by improving soil fertility and plant health (Ma *et al.*, 2024).

S. persica L. relies on nitrogen fixing bacteria for acquisition of nitrates (NO₃⁻) which is provided by *Rhizobium* spp. and *Azospirillum* spp. which convert atmospheric nitrogen (N₂) into ammonia (NH₃) and then ammonium (NH₄⁺). Afterward *Nitrosomonas* spp. and *Nitrobacter* spp. bacteria produce firstly nitrites (NO₂⁻) and then nitrates (NO₃⁻) from the ammonium provided by nitrogen fixing bacteria. These nitrates and their absorption are crucial for physiological functions of plants such as amino acid synthesis, protein and nucleic acid production (Robertson and Groffman, 2015). Thus, rhizospheric microorganism activity enhances the soil fertility, thus

promoting sustainable agriculture making the use of artificial nitrogenous fertilizers dispensable (Igiehon and Babalola, 2018; Harindintwali *et al.*, 2021). Within the agricultural frameworks, these bacterial communities dare a lot of attention for their habitat regulation, encouraging a sustainable eco-friendly strategy for yield enhancement (Mendes *et al.*, 2013; Priyadharsini *et al.*, 2016).

The ecological relevance of rhizobacterial communities in symbiotic relation with *S. persica* L. are crucial for our understanding in development of conservation of ethnobotanically profound species. These bacterial communities synergistically enhance and stabilise the growth and physiological aspects of these plants (Sharma *et al.*, 2023). Fascinatingly the rhizospheric bacteria associated with *S. Persica* L. have been analyzed to carry antimicrobial potential, an example of which is *Pseudomonas syringae* creating a symbiotic immunity for the plant (Mehmood *et al.*, 2023).

Biomolecular identification methods, particularly 16S rRNA gene sequencing, are crucial for identification and characterization of rhizobacterial communities in accordance with standardized identification, as culture dependent methods fail on various parameters. Thus, identification is the first step for unlocking the true potential of a biological factor (Clarridge *et al.*, 2004). While various reports and documentations have been made for the presence of floral diversity in the archaeological site of Harappa in Punjab, Pakistan (Farooqui *et al.*, 2013), the rhizobacterial diversity associated with the *S. persica* L. in this archeologically important site is yet completely un-investigated. Therefore, this study aimed to isolate, molecular identification and characterization of putative PGPR from the rhizome of *S. persica* L. acquired from Harrappa, Punjab. The objective is to treat strains from this unique environment, which could serve a multifaceted utilization firstly as a potential bioinoculants for conservation and cultivation of this *S. persica* L., as well as pharmaceutical drug design and sustainable agriculture.

MATERIALS AND METHODS

Study Site and Sample Collection

Considering temperature sensitivity, eight samples of rhizospheric soil of *S. persica* L. were collected at 5-20 cm of depth during two temperatures 27- 42°C in the July 2023 and 5-21°C in the month of October 2023, in the Harappa region, Sahiwal District, Punjab, Pakistan. Then the topsoil was removed with caution on the soil in close contact with the fine roots using a sterile trowel. A number of sub samples were cumulative product in order to obtain a composite sample of approximately 200 - 500 g in a sterile plastic bag. These samples were carried to the Plant Molecular Biology Lab, Department of Botany, Government College University, Lahore, Pakistan for further processing.

Bacterial Isolation and Pure Culture

LB Agar Media Preparation

Preparation of Luria-Bertani (LB) agar medium was conducted under the described methodology of MacWilliams and Liao (2006). 8g of Agar, 2.5g of Yeast Extract, 5g of Tryptone, and 5g of NaCl were thoroughly dissolved in 250 ml of distilled water. Then the medium was autoclaved at 121°C for 15 minutes for sterilization. Post autoclaved media was poured under aseptic conditions into the petri dishes and left to harden. Petri dishes were left at 37°C overnight to ascertain the lack of contamination

A volume of 1 g of rhizospheric soil was suspended in 9 mL of sterile distilled water to establish a 10⁻¹ dilution. Serial dilutions were subsequently prepared up to 10⁻⁶. From the 10⁻⁵ and 10⁻⁶ dilutions, an aliquot of 100 µL was surface spread onto LB agar plates in triplicate. The plates were incubated at 37°C for 24 - 48 hours. Following incubation, distinct bacterial colonies on the basis of morphological characteristics were picked and purified by sub-culturing on fresh LB agar plates through streaking. A single purified isolate was thereby obtained for further analysis and was stored in 20% glycerol at -20°C.

Morphological and Biochemical Characterization

Gram Staining

Gram staining was conducted according to the standard procedure by Smith and Hussey (2005). The bacterial smear was heat fixed, then sequentially stained with crystal violet for 1 minute, Gram's iodine for 1 minute, decolorized with 95% ethanol, and counterstained with safranin for 45 seconds. Observation of the Gram stained cells was made on a light microscope at 100X magnification under oil immersion.

Genomic DNA Extraction

The genomic DNA was extracted from a fresh pure culture using the modified phenol-chloroform method as described by John *et al.* (2023). In brief, bacterial cells were harvested from a plate and suspended in a 1X TE

buffer. Centrifugation at 4,000g for 15 min pelleted the cells, which were then resuspended in 450 μ L of TE buffer. The suspension was treated with 30 μ L of lysozyme (10 mg/mL) and incubated at 37°C for 15 min. Thereafter, 60 μ L of 10% SDS was added and incubation continued at 60°C for 15 min. Protein removal was achieved with the addition of an equal volume of chloroform-isoamyl alcohol (24:1) followed by centrifugation at 15,000g for 25 min. The aqueous upper phase containing the DNA was transferred to a new tube and DNA precipitated with three volumes of chilled 70% ethanol. After air drying, the DNA pellet was suspended in 50 μ L of the TE buffer. The extracted DNA was then stored at -20°C.

DNA Quantification

To assess the purity and concentration of the extracted DNA, electrophoresis was carried out on a 1% agarose gel with Ethidium Bromide staining and then visualized under ultraviolet light.

Amplification of the 16S rRNA Gene by PCR

Amplification of 16S rRNA gene was conducted by employing universal primers. Initial PCR parameters were determined by gradient PCR to test the annealing temperatures from 40°C to 60°C. The reaction mixture, in total 25 μ L, contained 12 μ L of PCR master mix, 5 μ L of each primer (forward and reverse; 10 μ M), 5 μ L of template DNA, and 7 μ L of nuclease free water. Amplification was carried out in a thermal cycler under the following conditions: initial denaturation at 94°C for 5 min; 35 amplification cycles of 94°C for 30 sec, 53°C for 1 min, 72°C for 1 min, and then final extension at 72°C for 7 min.

Gel Electrophoresis of PCR Products

The PCR products were then subjected to electrophoretic analysis on a 1% agarose gel made in 1X TAE buffer, running at 90 V for 40 minutes. After separation, the gel was stained with ethidium bromide and observed under UV light to confirm the expected size of the amplicon.

Sequencing and Phylogenetic Analysis

The PCR product was subsequently purified and sequenced with Sanger sequencing. The determined sequence of the 16S rRNA gene was subjected to NCBI BLAST in order to assess its taxonomic affiliation. A phylogenetic tree was subsequently drawn to confirm the evolutionary relationships of the isolate.

RESULTS

Isolation and Morphological Characterization of Rhizobacteria

Rhizospheric soil samples were acquired from five individual *S. persica* L. plants (Fig.1) at a sampling site in Harappa, Pakistan. Serial dilution and plating on Luria-Bertani (LB) agar led to the isolation of multiple bacterial colonies. Repeated streaking was performed to obtain pure cultures. Subsequent Gram staining of a dominant isolate revealed purple colored, rod shaped cells, confirming it as a Gram positive *Bacillus* (Fig.2). The consistent morphology across subcultures indicated the isolation of a uniform bacterial strain.



Fig. 1 Soil samples collected from the rhizosphere of *S. persica* L.

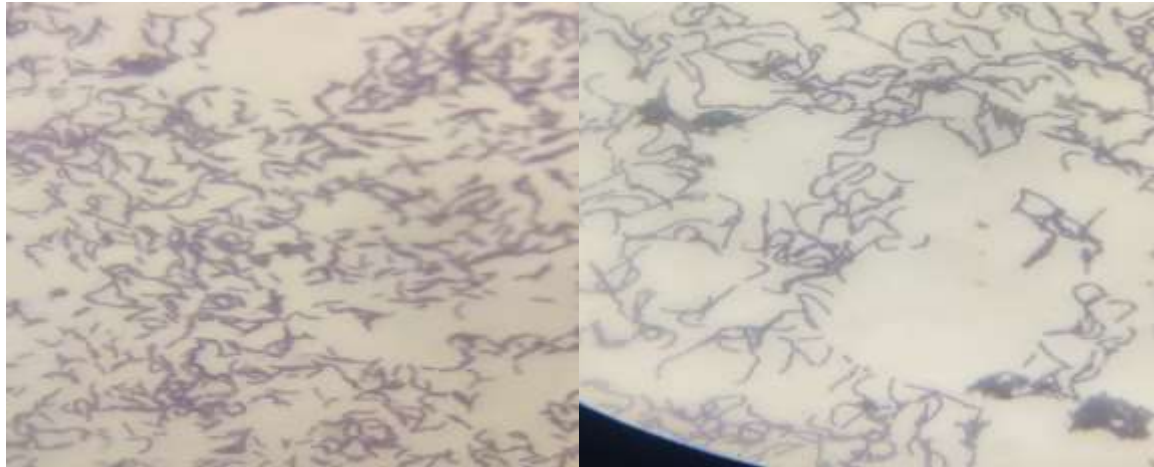


Fig. 2. Gram staining results showing rod-shaped, Gram-positive *bacillus* bacteria.

DNA Extraction and PCR Amplification for Molecular identification

Genomic DNA was successfully extracted from the pure culture using the modified phenol-chloroform-isoamyl alcohol method. The integrity and presence of genomic DNA were confirmed by agarose gel electrophoresis. Well denoted by L is having 1kb ladder while well No. 1, 2, 3, 4, 5, 6, and 7 contain DNA samples. Wells 1, 2, 4 and 5 showed clear, high molecular weight bands under UV illumination as shown in the Figure 3.

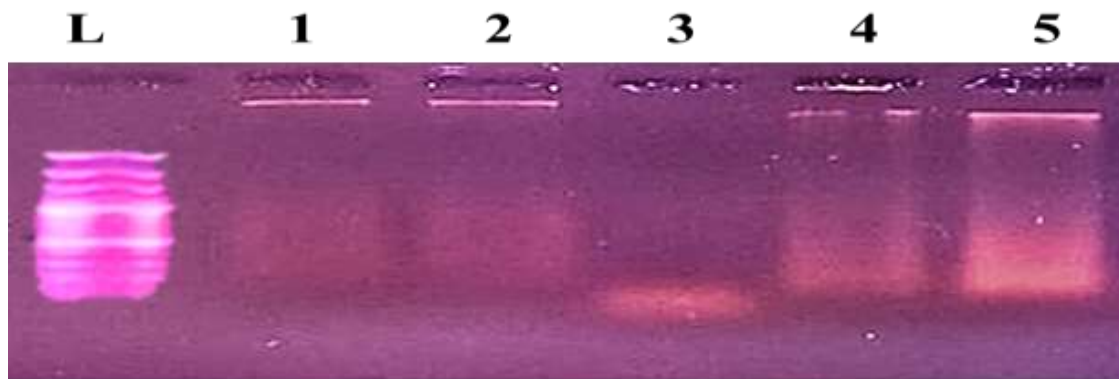


Fig.3. Agarose Gel electrophoresis results of isolated DNA.

To optimize the 16S rRNA gene amplification, a gradient PCR was carried out with annealing temperatures ranging from 40°C to 60°C. Agarose gel electrophoresis of the resultant PCR products on a 1.5% agarose matrix identified 53°C as the optimal annealing temperature, at which a single, specific amplicon of the expected size was produced with the least non-specific amplification. At 53 °C annealing temperature DNA samples were further amplified and further confirmed by agarose gel electrophoresis as shown in the Figure 4. Well L contain ladder while 1 to 9 contain amplicons. Well No. 1, 2, 3, 4, 5, 6, 7 and 9 showed fine bands of amplicons.

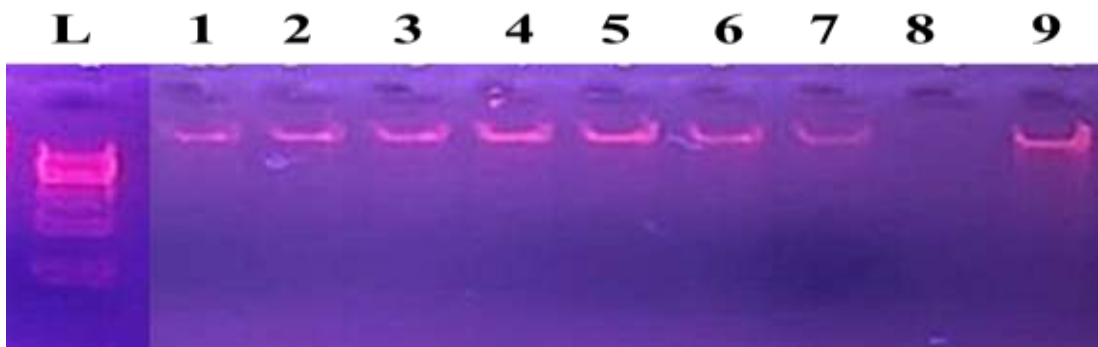


Fig. 4. PCR amplification results as seen on agarose gel electrophoresis.

Under these optimized conditions, a standard PCR was performed. Electrophoretic analysis of the resulting product confirmed that an approximately 898 bp fragment of the 16S rRNA gene had been successfully amplified as shown in the Figure 5.



Fig. 5. Agarose gel electrophoresis results of PCR products.

Gene Sequence and Phylogenetic Analysis

The purified PCR amplicon was sequenced and generated a sequence of 898 bp, which was submitted to the NCBI BLAST analysis tool. It showed 100% similarity with *Priestia megaterium* strains present in the GenBank database, which gave sufficient evidence of the fact that the bacterium isolated in this study is indeed *Priestia megaterium*.

To further confirm the taxonomic position of the isolate, a phylogenetic tree was constructed by using the Maximum Likelihood method with the Tamura-Nei model by using MEGA11. The analysis involved 10 sequences and covered 990 positions within the final dataset. The sample sequence formed a strong cluster with reference sequences of *Priestia megaterium*, further confirming its assignment to this species (Fig. 6).

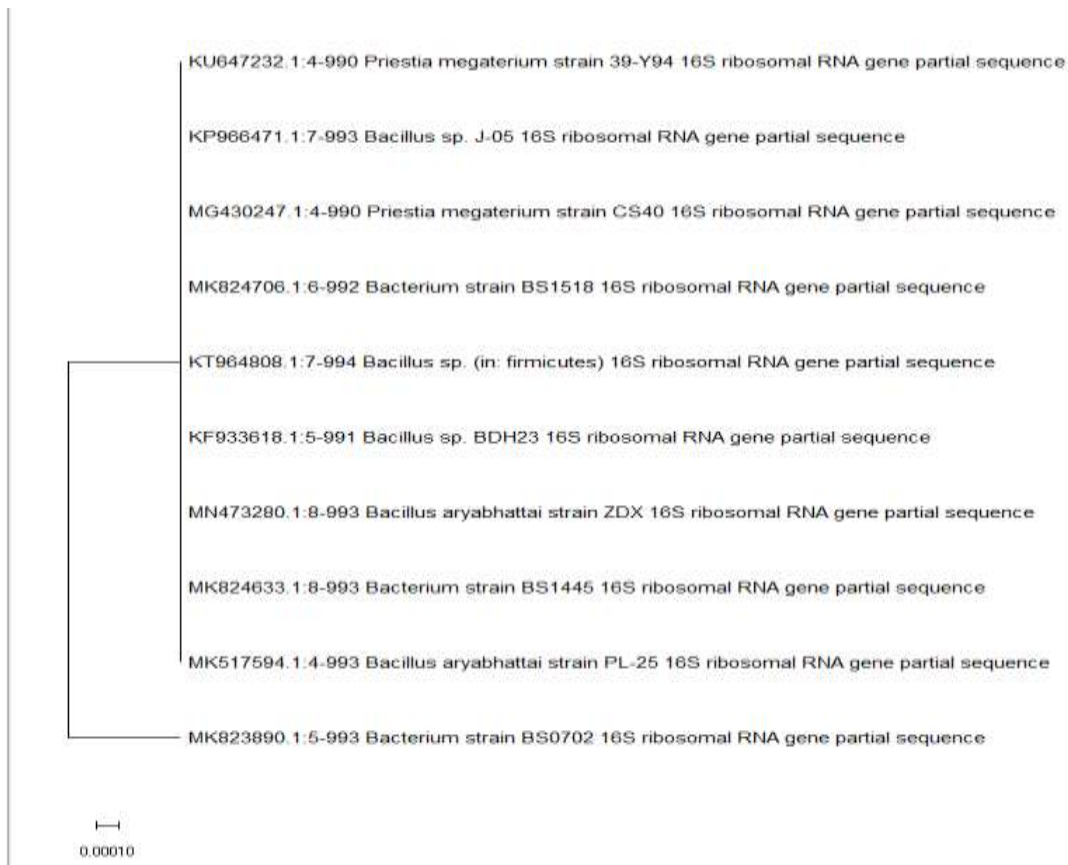


Fig. 6. Phylogenetic analysis of *P. megaterium*.

DISCUSSION

The rhizosphere comprises a complex microbial assemblage, which is considered significant in determining the health of a plant and soil fertility. Plant Growth Promoting Rhizobacteria (PGPR) are part of this microbial group that improves the plant development using multiple mechanisms in relation to the production of phytohormones, nutrient solubilization, and induction of systemic resistance against pathogens or abiotic stresses (Khosso *et al.*, 2024). The current study has been focused on the isolation and molecular identification of rhizobacteria associated with *S. persica* L., which is an ethnopharmacologically important yet endangered plant from the Harappa region of Pakistan.

Using culture dependent methods, the dominant bacterium isolated was Gram-positive and rod shaped. Such morphology is typical of several beneficial rhizospheric *Bacilli* (Radhakrishnan *et al.*, 2017). Traditionally Identification depended on phenotype and biochemical tests, while modern practice targets molecular approaches for a more specific taxonomic resolution. In this regard, we employed 16S rRNA gene sequencing, which has become a popular method for the identification of bacteria because of the presence of conserved and variable regions within this gene.

Accordingly, high quality genomic DNA was extracted and the 16S rRNA gene amplified by PCR. BLAST analysis of the resulting 898 bp sequence indicated 100% similarity to *Priestia megaterium*. This is noteworthy since *Priestia megaterium*, previously classified under the genus *Bacillus*, has been widely recognized as a plant growth promoter. Its presence in the rhizosphere of *S. persica* L. points toward a possible symbiotic role that improves the plant's fitness in its native semi - arid environment. *Priestia megaterium*, along with related *Bacilli*, possesses multifunctional PGPR characteristics. They can act as biocontrol agents through the production of antibiotics and siderophores, compete with pathogens for nutritional gains, and even induce systemic resistance in plants (Zhu *et al.*, 2025). Besides this, their ability to produce phytohormones and solubilize growth limiting nutrients like phosphorus and potassium, can enhance direct plant growth and soil health (Babar *et al.*, 2023). Beyond agricultural uses, *Priestia megaterium* has a lot of potential in biotechnology, starting from industrial enzymes production up to the strategies of bioremediation, including heavy metal detoxification, and might be considered a model probiotic source (Oziegbe *et al.*, 2024). The isolation of *Priestia megaterium* from *S. persica* L. indicates the ecological relevance of rhizobacterial populations to the survival of plant species, especially those that are endangered under harsh conditions. This may provide a contribution to the resilience of *S. persica* L. by improving nutrient uptake and overcoming environmental stresses.

CONCLUSION

A prominent rhizobacterium was obtained from the rhizosphere of *S. persica* L. and was found to be *Priestia megaterium* based on the gene sequencing of the 16S rRNA of the bacterium. The detection of this well-known PGPR associated intimately with *S. persica* L. indicates the potential importance of associated microbes in the adaptability of this plant species under the native environment. The results of current study open channels for future research aimed at isolating the distinct growth-promoting factors of this microbe, such as the production of phytohormones and phosphate solubilization. The exploitation of this environment friendly, useful microbe could provide a feasible avenue for the preparation of biofertilizers and biocontrol agents useful for the conservation of endangered plants like *S. persica* L. as well as creating a paradigm shift towards more environment friendly farming practices.

AUTHOR CONTRIBUTION

Project conceptualization and supervision was done by Muhammad Umer Farooq Awan. Sample collection, conduction of analyses and first draft of the manuscript was written by Abdullah Qasim. All the contributing authors commented and edited before submission. Final draft was written by Wasim Abbas, Ali Hasnain and Yousaf Qaiser.

CONFLICT OF INTEREST

The authors have no relevant financial or non-financial conflict of interest to disclose.

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