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Characterization of Phosphate-Solubilizing Fluorescent Pseudomonads from the Rhizosphere of Seabuckthorn Growing in the Cold Deserts of Himalayas

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Abstract Isolation and characterization of fluorescent pseudomonads with high phosphate-solubilizing ability is reported from the alkaline and calcium-rich soils with low P availability in the cold desert region of Lahaul and Spiti in the trans-Himalayas of India. Of 216 phosphate-solubilizing isolates, 12 exhibiting high solubilization of tricalcium phosphate (TCP) in NBRIP liquid culture were identified as *Pseudomonas trivialis*, *P. poae*, *P. fluorescens*, and *Pseudomonas* spp. on the basis of phenotypic features, whole-cell fatty acids methyl ester (FAME) profiles, and 16S rDNA sequencing. These isolates also showed relatively high solubilization of North Carolina rock phosphate (NCRP) in comparison to the solubilization of Mussoorie rock phosphate (MRP) and Udaipur rock phosphate (URP). The solubilization of phosphate substrates by *P. trivialis* and *P. poae* is reported for the first time.

Introduction

Phosphorus is an essential mineral nutrient that often limits plant growth because of its low solubility and fixation in the soil. The release of fixed and poorly soluble forms of phosphorus is an important aspect for increasing soil fertility. Phosphate-solubilizing microorganisms bring about mobilization of insoluble phosphates in the soil and increase plant growth under conditions of poor phosphorus availability [31]. The phosphate solubilization ability of microorganisms has been employed for improving crop yield in agriculture and horticulture [13, 25]. These microorganisms also hold the potential of ecological amelioration of degraded and disturbed forest wastelands by improving growth and establishment of plants under low phosphorus availability. Fluorescent pseudomonads often predominate among bacteria in the plant rhizosphere [30]. These beneficial bacteria enhance plant growth by improving soil nutrient status, secreting plant growth regulators, and suppressing soil-borne pathogens [14].

Located between latitude 31° 42′ 33″ N and longitude 77° 21′ 35″ E in the Indian trans-Himalayas, the cold desert of Lahaul and Spiti is marked by a rugged and fragile mountainous terrain with poor availability of some essential mineral nutrients in the soil [28]. Consequently, these valleys have a sparse vegetation cover. Application of nursery plants with suitable native microbial inoculants appears promising through afforestation practices to aid in the restoration of degraded landscape. Information on the occurrence and activity of phosphate-solubilizing microorganisms is lacking for the cold desert soils.

Seabuckthorn (*Hippophae rhamnoides* L.) is ecologically an important plant with the highest Importance Value Index in Lahaul and Spiti [28]. Plantation of the spinescent shrub-tree has been taken up in the valleys of the fragile mountainous system to avoid erosion and degradation. This article reports the screening and characterization of phosphate-solubilizing fluorescent pseudomonads from the rhizosphere of seabuckthorn as a first step to developing microbial additives for application in afforestation procedures.

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Materials and Methods

Soil Sampling and Isolation of Fluorescent Pseudomonads

Soil samples were collected from the rhizosphere of feeder roots of the natural populations of *Hippophae rhamnoides* growing at Rong Tong at an elevation of about 3900 m above mean sea level in Lahaul and Spiti in the trans-Himalayan region. A total of six composite soil samples were used for isolation of fluorescent pseudomonads. Each composite sample comprised the equal quantities of nine air-dried and thoroughly mixed soil samples retrieved from the rhizosphere of three plants. Fluorescent pseudomonads were isolated by plating serial soil dilutions up to 10^{-4} on King's B medium, and fluorescent colonies detected under UV light (302–365 nm) were purified on the same medium [15].

Selection of Efficient Phosphate-Solubilizing Fluorescent Pseudomonads

Bacterial isolates were evaluated for their ability to solubilize inorganic phosphate by spotting the inoculum on modified Pikovskaya (PVK) agar and measuring the size of the phosphate solubilization halo around the colonies after 7 days of incubation at 28°C [8].

Quantitative estimation of inorganic phosphate solubilization was done in NBRIP broth [18] containing 0.5% tricalcium phosphate (TCP), Mussoorie rock phosphate (MRP), Udaipur rock phosphate (URP), or North Carolina rock phosphate (NCRP). The rock phosphates were washed to remove soluble phosphorus and dried at 40°C for 24 h before their use as phosphate substrates. The 250-mL flasks containing 50 mL NBRIP medium inoculated with 500 µL bacterial culture (inoculum adjusted $\sim 5 \times 10^8$ CFU/mL) were incubated at 28°C in a refrigerated incubator shaker at 180 rpm for 5 days. The phosphorus content in culture filtrates was estimated by the vanado-molybdate method [11]. The uninoculated autoclaved media with different phosphate substrates incubated under a similar set of conditions as the inoculated cultures were used as the controls. Values of P-liberated are expressed as µg/mL over the control. The pH of liquid medium was measured in each case.

Phenotypic Characterization of Bacterial Isolates

Phenotypic characterization of isolates was done based on their colony morphology, microscopic observations, and biochemical tests [9]. The isolates were tested for the utilization of 35 carbon sources using HiCarbohydrate™ Kit (HiMedia, Mumbai). The results of the phenotypic characters of the bacterial isolates were scored as negative (0) or positive (1). A dendrogram was constructed based on the matrix generated by the phenotypic characters using TREECON software v1.3b (Yves Van de Peer, University of Antwerp) (Fig. 1).

Whole-Cell Fatty Acids Methyl Ester (FAME) Analysis

The isolates were identified based on whole-cell fatty acids, derivatized to methyl esters, and analyzed by gas chromatography using the Sherlock Microbial Identification System (MIS-MIDI, USA). The FAME profiles were compared with the TSBA50 aerobe library general system software v5.0. Qualitative and quantitative differences in the fatty acid profiles were used to compute the distance for each strain relative to the strains in the library [26, 27].

16S rDNA Sequence Analysis

Genomic DNA was extracted by using Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Amplification of 16S rDNA was performed using fD1 (5′-AGAGTTTGA TCCTGGCTCAG-3′) and rP2 (3′-ACGGCTACCTTGTT ACGACTT-5′) primers [33]. The total volume of PCR reaction mixture was 50 µL, comprising 200 µM dNTPs, 50 µM each primer, 1× PCR buffer, 3 U *Taq* polymerase, and 100 ng genomic DNA. The thermocycling procedure involved an initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min, and final extension at 72°C for 8 min. The gel-purified 16S rDNA was ligated to pGEM-T easy vector (Promega, Madison) and transformed in *E. coli* JM109. The sequences of the insert were determined using a Big-Dye Terminator Cycle Sequencer and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA). The

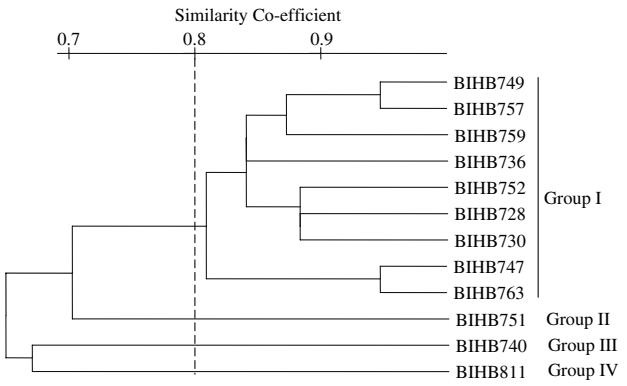

Fig. 1 Dendrogram of phosphate-solubilizing fluorescent pseudomonads isolates based on the similarity coefficient derived from their phenotypic characters

sequences were analyzed using the gapped BLASTn (http://www.ncbi.nlm.nih.gov) search algorithm and aligned to their nearest neighbors. The evolutionary distances among phosphate-solubilizing isolates and their related taxa were calculated using TREECON software and Kimura's two-parameter model, after aligning the sequences with ClustalW. The 16S rDNA sequence of *Burkholderia graminis* C4D1M^T was used as an outgroup. The cultures have been deposited in the Microbial Type Culture Collection and Gene Bank at the Institute of Microbial Technology, Chandigarh, India.

Data Analysis

The data were subjected to two-way analysis of variance (ANOVA) using STATISTICA data analysis software v7 (StatSoft Inc., Tulsa, OK). All values are the means of three replicates and repeated twice. The means of the treatments were compared by CD value at $p = 0.01$. For analysis of data of GC-FAME profiles, a joining method of the cluster analysis (CA) module (Statistica 7) and the clustering algorithm of Ward were applied. This way a dendrogram was generated showing clustering trends among the fluorescent pseudomonads (Fig. 3).

Nucleotide Accession Numbers

The 16S rDNA sequences of phosphate-solubilizing fluorescent pseudomonads (~1500 bp) were deposited in the NCBI GenBank database under the accession numbers (isolates): DQ 536512 (BIHB 728), DQ 536513 (BIHB 730), DQ 536514 (BIHB 736), DQ 536515 (BIHB 740), DQ 536517 (BIHB 747), DQ 536518 (BIHB 752), DQ 536519 (BIHB 757), DQ 536520 (BIHB 759), DQ 536521 (BIHB 751), DQ 885947 (BIHB 763), DQ 885949 (BIHB 749), and DQ 885950 (BIHB 811).

Results and Discussion

In the present study, 367 isolates of fluorescent pseudomonads were isolated by plating soil dilutions on King's B agar, of which 216 isolates showed the development of sharp phosphate solubilization zones, ranging from 5 to 22 mm on modified PVK agar. The other isolates showed the development of hazy zones. Twelve isolates producing zones greater than 10 mm were selected for quantification of phosphate solubilization of different inorganic phosphates.

The results show the solubilization of different phosphate substrates by the bacterial isolates. The solubilization of TCP was significantly higher than the solubilization of NCRP, MRP, and URP (Table 1). Among the rock phosphates, the solubilization of NCRP was significantly higher than the solubilization of MRP and URP. No significant difference was recorded in the solubilization of MRP and URP. The high solubilization by these isolates of NCRP as compared to that of MRP and URP corroborated the earlier report on the solubilization of these rock phosphates by *P. striata* and *B. polymyxa* [23]. The solubilization of rock phosphates has been reported to depend on their structural complexity and particle size as well as on the nature and

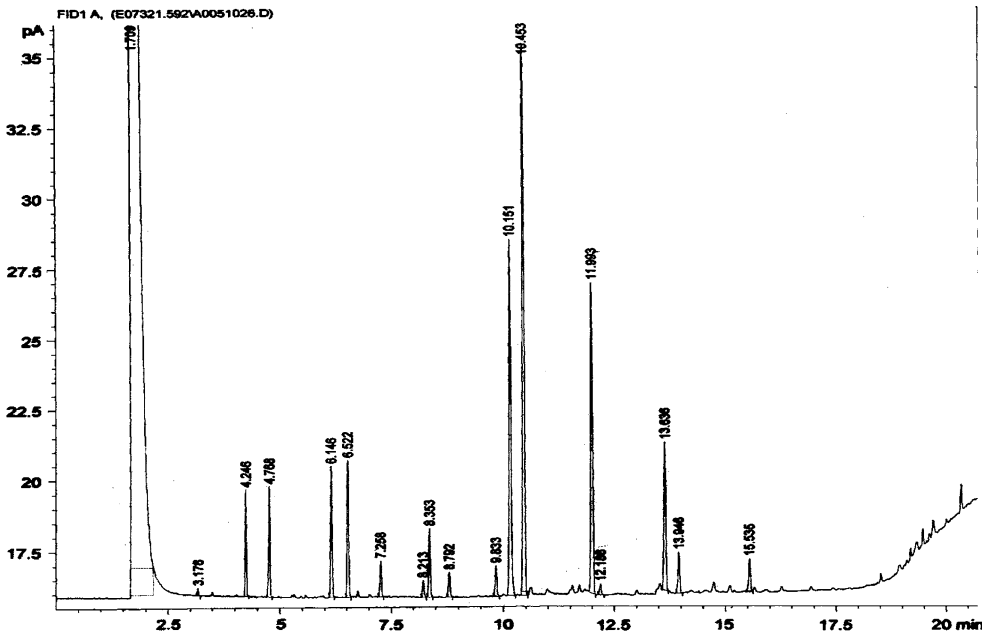

Fig. 2 Chromatograph showing the peaks of the FAME analysis for the isolate BIHB 728

quantity of organic acids secreted by the microorganisms [7]. The bacterial isolates differed in their ability to solubilize various phosphate substrates. The maximum solubilization was observed with the isolate BIHB 752 and the minimum solubilization with the isolate BIHB 751. These isolates appear to be among the most efficient phosphate-solubilizing bacterial strains in comparison with the earlier reports on microbial solubilization of phosphate substrates. The TCP solubilization by these isolates ranged from 319 to 805 μg/mL compared with 450, 290, 250, and 200 μg/mL exhibited by the four most efficient bacterial isolates NBRI2601, NBRI3246, NBRI0603 and NBRI4003, respectively, from the alkaline soils of tropical India [19]. Johri et al. [12] have reported 510-μg/mL TCP solubilization by the best bacterial strain from the alkaline soils. Likewise, TCP solubilization ranged from 96 to 139 μg/mL by the 13 best isolates clustered under the genera *Enterobacter*, *Pantoea*, and *Klebsiella* from Korean soils [5]. Recently, the soluble phosphate production around 900 μg/mL has been reported for *Pantoea agglomerans* isolated from the acidic soils of Korea [29].

A significant decline in the pH of medium was recorded during the solubilization of different phosphate substrates, which suggested secretion of organic acids by the bacterial isolates [4, 10, 31]. The release of soluble phosphates is not necessarily correlated with acidity [2]. In the present study no relationship could be ascertained between the quantity of phosphate solubilization and acidity of medium because decline in pH during solubilization of TCP and NCRP was at par, though solubilization was significantly higher for TCP than for NCRP. Likewise, the decrease in pH was not always correlated with the quantity of phosphate solubilized, because the decline in pH was disparate with the quantity of phosphate solubilization by some isolates (Table 1). Earlier findings on the solubilization of rock phosphates by some efficient microbial strains also indicated that phosphate solubilization varies greatly with the nature of phosphate substrates and the organisms [3].

The phosphate-solubilizing bacterial isolates showed differences in their morphologic and biochemical characteristics. The colony morphology of the isolates varied from circular to flat, convex, or raised, margins entire or undulating. All isolates were Gram-negative, nonendospore-forming, and motile rods; positive for catalase, oxidase, and aerobic acid formation from glucose, but indole-negative; and exhibited the characteristic green fluorescence under UV light on King's B medium. These characteristics placed the isolates in the group fluorescent pseudomonads. The strains belonging to the fluorescent pseudomonads are among the common inhabitants of the rhizosphere involved in several interactions with plants [24]. Majority of the isolates were negative for urease and positive for gelatin hydrolysis. All isolates were positive for the utilization of dextrose, citrate, malonate, glycerol,

Table 1 Solubilization of phosphate substrates by fluorescent pseudomonads in NBRIP broth at 28°C

Isolate	Phosphate solubilization over control (μg/ml)					Final pH of the medium[a]				
	TCP	NCRP	MRP	URP	Mean	TCP	NCRP	MRP	URP	Mean
BIHB 728	771.3	191.3	11.0	8.7	245.6	3.63	3.70	3.52	3.78	3.66
BIHB 730	768.3	163.8	4.0	5.0	235.3	3.40	3.90	4.62	3.70	3.90
BIHB 736	778.7	172.0	13.1	5.6	242.3	3.90	3.72	3.52	3.79	3.73
BIHB 740	768.3	236.8	3.0	3.8	253.0	3.97	3.48	5.90	4.00	4.34
BIHB 747	743.0	173.0	12.0	4.4	233.1	3.52	3.81	3.49	3.71	3.63
BIHB 749	801.0	177.3	8.0	5.3	247.9	3.42	3.73	3.59	3.60	3.58
BIHB 751	318.7	123.3	2.4	1.4	111.4	4.20	3.89	3.89	4.20	4.05
BIHB 752	805.0	204.3	6.0	7.7	255.8	3.50	3.72	3.62	3.90	3.68
BIHB 757	775.3	175.0	9.0	7.1	241.6	3.92	3.92	3.63	3.72	3.80
BIHB 759	751.3	178.0	11.0	14.0	238.6	3.72	3.81	3.52	3.62	3.66
BIHB 763	718.0	161.2	12.9	9.3	225.3	4.00	3.80	3.50	3.78	3.77
BIHB 811	717.3	173.0	2.9	6.1	224.8	3.98	3.92	4.00	4.11	4.00
Mean	726.4	177.4	7.9	6.5		3.76	3.78	3.90	3.83	
Variant	SEM ±			CD (p = 0.01)		SEM ±		CD (p = 0.01)		
P Source	0.3			1.2		0.01		0.05		
Isolates	0.6			2.2		0.02		0.08		
Interactions	1.2			4.3		0.04		0.16		

TCP = tricalcium phosphate; NCRP = North Carolina rock phosphate; MRP = Mussoorie rock phosphate; URP = Udaipur rock phosphate

[a] Initial pH 6.8

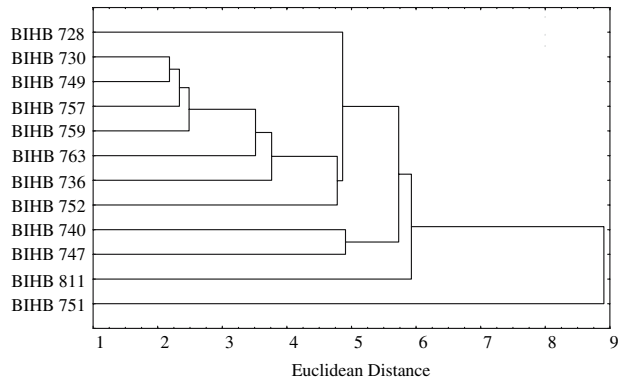

Fig. 3 Dendrogram representing the whole-cell fatty acid compositional similarity of fluorescent pseudomonads strains obtained by GC-FAME

melibiose, L-arabinose, D-arabinose, mannose, ribose, and xylose, but negative for the utilization of maltose, adonitol, esculin, ONPG, sodium gluconate, salicin, glucosamine, dulcitol, sorbose, xylitol, α-methyl-D-glucoside, D-mannoside, and melezitose. The majority of the isolates were also positive for the utilization of cellobiose, rhamnose, and galactose. However, the utilization of other carbon sources was limited to only some isolates—lactose by seven isolates, raffinose by four isolates, fructose and sucrose by three different isolates, sorbitol by two isolates, and inulin, inositol, and mannitol by single isolates. The similarity dendrogram based on the above characteristics showed one major cluster of nine isolates at an 80% similarity level (Fig. 1). The remaining three isolates stood independently outside the major cluster, mainly due to the differences in the utilization of carbon sources.

FAME analysis showed subtle differences in the composition of cell-wall fatty acids of various isolates. The fatty acids 16:0, 16:0 w7c/15 iso 2OH,17:0 cyclo, 10:0 3 OH, 12:0, 12:0 2 OH, 12:0 3 OH, 14:0, and 18:1 w7c were present in all the isolates. Small amounts of 10:0, 17:0, 18:1 iso H, 18:0, 19:0 iso, and 19:0 cyclo w8c were also detected in some strains, while the fatty acids 15:0 iso, 15:0 anteiso, and 16:0 iso were obtained for only one isolate (Fig. 2). The analysis showed the highest similarity of all isolates with *Pseudomonas putida*, except one isolate that showed the highest similarity with *Aquaspirillum autotrophicum* (Table 2). Each isolate exhibited a specific fatty acid composition, making it a "microbial fingerprint." The cluster analysis produced two groups consisting of eight isolates and two isolates, while the two isolates stood independently outside these clusters. The FAME analysis dendrogram resembled the groupings based on phenotypic characters, with the exception of isolate BIHB 747 which grouped with the isolate BIHB 740 and not the major cluster (Fig. 3).

The results of a BLAST search of 16S rDNA sequences of the fluorescent pseudomonads compared with the available 16S rDNA sequences in the GenBank database indicated that *Pseudomonas trivialis* DSM 14937^T was the closest related species to the seven isolates (Table 2). The sequences of two isolates BIHB 730 and BIHB 752 showed maximum similarity with *Pseudomonas poae* DSM 14936^T, while the other three isolates BIHB 740, BIHB 751, and BIHB 811 were closely related to *Pseudomonas fluorescens* Pf29A, *Pseudomonas* sp. A-13, and *Pseudomonas* sp. P 12, respectively. The phylogenetic tree based on 16S rDNA sequences of the isolates and representative species of the genus *Pseudomonas sensu stricto* [1] formed three clearly distinguishable groups (Fig. 4). The first group included nine isolates along with *Pseudomonas trivialis* DSM 14937^T, *Pseudomonas poae* DSM 14936^T, and *Pseudomonas antarctica* strain CMS 35^T. The second group consisted of the isolates BIHB 740, BIHB 811,

Table 2 Identification of phosphate-solubilizing fluorescent pseudomonads by GC-FAME and 16S rDNA sequencing

Isolate	GC-FAME identification	Similarity (%)	16S rDNA identification	Identity (%)
BIHB 728	*Pseudomonas putida*	7.7	*Pseudomonas trivialis*	99.6
BIHB 730	*Aquaspirillum autotrophicum*	47.7	*Pseudomonas poae*	99.9
BIHB 736	*Pseudomonas putida*	15.3	*Pseudomonas trivialis*	99.6
BIHB 740	*Pseudomonas putida*	18.3	*Pseudomonas fluorescens*	99.7
BIHB 747	*Pseudomonas putida*	36.5	*Pseudomonas trivialis*	99.8
BIHB 749	*Pseudomonas putida*	36.3	*Pseudomonas trivialis*	99.8
BIHB 751	*Pseudomonas putida*	15.9	*Pseudomonas* sp. A-13	99.3
BIHB 752	*Pseudomonas putida*	21.5	*Pseudomonas poae*	99.7
BIHB 757	*Pseudomonas putida*	39.8	*Pseudomonas trivialis*	99.7
BIHB 759	*Pseudomonas putida*	30.3	*Pseudomonas trivialis*	99.7
BIHB 763	*Pseudomonas putida*	30.1	*Pseudomonas trivialis*	99.9
BIHB 811	*Pseudomonas putida*	51.2	*Pseudomonas* sp. P 12	99.9

Fig. 4 Phylogenetic tree based on 16S rDNA sequences drawn using the neighbor-joining method, showing the relationships between fluorescent pseudomonads isolates and species from the genus *Pseudomonas sensu stricto* with validly published names. The 16S rDNA accession numbers are given within brackets. Bar = 0.02 substitutions per site

Pseudomonas kilonensis 520-20, and *Pseudomonas corrugata* ATCC 29736^T. The third group included BIHB 751, *Pseudomonas putida* IAM 1236^T, *Pseudomonas rhizosphaerae* IH5^T, *Pseudomonas lutea* OK2^T, and *Pseudomonas graminis* DSM 11363^T.

A few species of the genus *Pseudomonas* such as *P. putida* [16], *P. aeruginosa* [17], *P. corrugata* [20], *P. lutea* [22], *P. fluorescens* [6], *P. rhizosphaerae* [21], and *P. stutzeri* [32] are known to be phosphate solubilizers. The solubilization of inorganic phosphates has not been previously reported for *Pseudomonas trivialis* and *P. poae*. In the present study *P. trivialis* appears predominant among the phosphate-solubilizing fluorescent pseudomonads in the rhizosphere of seabuckthorn. These isolates with high phosphate-solubilizing ability appear attractive for exploring their plant growth-promoting activity toward the development of microbial inoculants.

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