Ann Microbiol (2015) 65:1885–1899
DOI 10.1007/s13213-014-1027-4

ORIGINAL ARTICLE

Assessment of genetic diversity and plant growth promoting attributes of psychrotolerant bacteria allied with wheat (*Triticum aestivum*) from the northern hills zone of India

Priyanka Verma · Ajar Nath Yadav · Kazy Sufia Khannam · Neha Panjiar · Sanjay Kumar · Anil Kumar Saxena · Archna Suman

Received: 1 October 2014 / Accepted: 17 December 2014 / Published online: 29 January 2015

Abstract The biodiversity of wheat-associated bacteria from the northern hills zone of India was deciphered. A total of 247 bacteria was isolated from five different sites. Analysis of these bacteria by amplified ribosomal DNA restriction analysis (ARDRA) using three restriction enzymes, *Alu*I, *Msp*I and *Hae*III, led to the grouping of these isolates into 19–33 clusters for the different sites at 75 % similarity index. 16S rRNA gene based phylogenetic analysis revealed that 65 %, 26 %, 8 % and 1 % bacteria belonged to four phyla, namely Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes, respectively. Overall, 28 % of the total morphotypes belonged to *Pseudomonas* followed by *Bacillus* (20 %), *Stenotrophomonas* (9 %), *Methylobacterium* (8 %), *Arthrobacter* (7 %), *Pantoea* (4 %), *Achromobacter*, *Acinetobacter*, *Exiguobacterium* and *Staphylococcus* (3 %), *Enterobacter*, *Providencia*, *Klebsiella* and *Leclercia* (2 %), *Brevundimonas*, *Flavobacterium*, *Kocuria*, *Kluyvera* and *Planococcus* (1 %). Representative strains from each cluster were screened in vitro for plant growth promoting traits, which included solubilisation of phosphorus, potassium and zinc; production of ammonia, hydrogen cyanide, indole-3-acetic acid and siderophore; nitrogen fixation, 1-aminocyclopropane-1-carboxylate deaminase activity and biocontrol against *Fusarium graminearum*, *Rhizoctonia solani* and *Macrophomina phaseolina*. Cold-adapted isolates may have application as inoculants for plant growth promotion and biocontrol agents for crops growing under cold climatic conditions.

Keywords Epiphytic · Endophytic · Rhizospheric · Northern hills zone · K-solubilisation

Priyanka Verma and Ajar Nath Yadav contributed equally to the present work.

Electronic supplementary material The online version of this article (doi:10.1007/s13213-014-1027-4) contains supplementary material, which is available to authorized users.

P. Verma · A. N. Yadav · A. K. Saxena · A. Suman (✉)
Division of Microbiology, Indian Agricultural Research Institute, New Delhi 110012, India
e-mail: archsuman@yahoo.com

P. Verma · K. S. Khannam
Department of Biotechnology, National Institute of Technology, Durgapur 713209, India

A. N. Yadav · N. Panjiar
Department of Bio-Engineering, Birla Institute of Technology, Mesra, Ranchi 835215, India

S. Kumar
Division of Genetics, Indian Agricultural Research Institute, New Delhi 110012, India

Introduction

An extreme environment of low temperature is one of the major limiting factors to plant growth and its productivity. Potentially plant growth promoting bacteria (PGPB) can enhance plant resistance towards biotic and abiotic stresses (Yang et al. 2009; Grover et al. 2011) and improve plant growth along with soil conditions (Tilak et al. 2005; Vyas et al. 2009). Tolerance to stress provided by microbial inoculants becomes more significant within the perspective of crop production losses due to severe abiotic stress (Grover et al. 2011). Wheat-associated bacterial diversity inhabiting low temperature environments has been investigated extensively in the past few years with a focus on culture-dependent techniques (Selvakumar et al. 2011). Many cold-tolerant PGPB

have been reported from low temperature environments included *Arthrobacter*, *Bacillus*, *Exiguobacterium*, *Pseudomonas* and *Providencia* (Mishra et al. 2011; Selvakumar et al. 2011; Bisht et al. 2013; Yadav et al. 2014a, b).

Quantitative and qualitative variations in plant growth promoting traits allow these bacteria to inhabit diverse niches in agro-ecosystems. The phyllosphere is a common niche for synergism between bacteria and plants. Microorganisms on leaf surfaces are extremophiles as they tolerate high temperature (40–55 °C) and UV radiation. *Agrobacterium*, *Methylobacterium*, *Pantoea* and *Pseudomonas* have been reported earlier in the phyllosphere (Holland et al. 2000; Verma et al. 2014). Rhizospheric bacteria have the ability to attach to root surfaces, allowing these to derive maximum benefit from root exudates (Bais et al. 2006). Endophytic bacteria live in plant tissues without causing substantive harm to the host. They exist within the living tissues of most plant species in a manner ranging from symbiotic association to slightly pathogenic. Such bacteria have been isolated from a variety of plants including citrus (Araujo et al. 2002), cotton (Misaghi and Donndelinger 1990), potato (Sturz et al. 1999), sweetcorn (McInroy and Kloepper 1995), sugarcane (Magnani et al. 2010), oilseed rape, tomato (Nejad and Johnson 2000), prairie and agronomic plants (Zinniel et al 2002). To the best of our knowledge, this is the first report of putative endophytes from wheat growing in a low-temperature region. *Achromobacter*, *Burkholderia*, *Microbiospora*, *Micromomospora*, *Nocardioides*, *Pantoea*, *Planomonospora*, *Pseudomonas*, *Streptomyces* and *Thermomonospora* have been isolated and characterized from internal tissue of wheat (Conn and Franco 2004; Jha and Kumar 2009; Verma et al. 2014). Epiphytic, endophytic and rhizospheric bacteria have been shown to promote plant growth directly, e.g. by fixation of atmospheric nitrogen, solubilisation of minerals such as phosphorus, potassium and zinc, production of siderophores and plant growth hormones such as cytokinin, auxin and gibberellins. Several bacteria support plant growth indirectly via production of antagonistic substances by inducing resistance against plant pathogens (Compant et al. 2005; Mishra et al. 2011; Verma et al. 2014).

Wheat (*Triticum aestivum* L.) is a major staple food crop for more than one-third of the world's population and is the main staple food of Asia. Wheat farming in the northern hills zone (NHZ) of India covers humid western Himalayan regions (Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Sikkim and hilly regions of West Bengal), and produces approximately 2 million tons of wheat with a rather low productivity level of 1.75 t ha^{-1} (Gupta and Kant 2012). The NHZ is characterised by its cold climatic and longer cultivating season for wheat crops. Low temperature is one of the major constraints hampering wheat production in the country. Hence, the present investigation deals with the enumeration, characterisation and phylogenetic profiling of bacteria associated with wheat cultivated in different parts of the NHZ of India.

Materials and methods

Study area and sample collection

Wheat plants with rhizospheric soil were collected from different parts of the NHZ of India, which included Jammu and Kashmir [Ranbirsingpora (32° 36′ 21″ N:74° 43′ 50″ E), Samba (32° 33′ 11″ N:75° 06′ 38″ E) and Riasi (33° 05′ 27″ N:74° 50′ 41″ E)], Himachal Pradesh [Shimla (31° 06′ 16″ N:77° 10′ 24″ E), Kangra (32° 05′ 59″ N:76° 16′ 09″ E), Mandi (31° 42′ 29″ N:76° 55′ 52″ E) and Sirmaur (30° 33′ 46″ N:77° 28′ 12″ E)], Uttarakhand [Almora (29° 27′ 12″ N:79° 40′ 12″ E) and Bageshwar (29° 50′ 22″ N:79° 46′ 35″ E)], Sikkim [east (27° 18′ 30″ N:88° 40′ 21″ E) and south (27° 17′ 09″ N:88° 23′ 40″ E) district] and hilly regions of West Bengal (23° 03′ 05″ N:87° 57′ 38″ E). A total of 60 samples, 12 from each site, was collected in sterile polythene bags, labelled, transported on ice and processed immediately.

Physico-chemical properties of samples

The pH and conductivity of the samples were recorded at the sampling site. Soil samples were analysed for soil organic carbon according to the rapid titration method of Walkley and Black (1934). Total nitrogen (%) was analysed using Kjeldahl's procedure using an N-analyzer UDK-149 (VELP Scientifica, Usmate Velate, Italy). Soil organic matter was determined by the loss of ignition method. Exchangeable cations (Ca, K, Mg and Na) were extracted with 1 M ammonium acetate (pH 7.0) and Ca, K and Na contents were determined with an atomic absorption spectrophotometer. Available phosphorus was determined by the Bray II method (Bray and Kurtz 1945). Soil analyses were performed at the Division of Soil Sciences, Indian Agricultural Research Institute, New Delhi, India.

Isolation and enumeration of wheat-associated bacteria

Culturable bacteria from the rhizospheric soils were isolated through an enrichment method, using a standard serial dilution plating technique. Ten different media were used to isolate the maximum possible number of culturable morphotypes (Supplementary Table 1). *Bacillus* sp. was isolated using a modified heat enrichment technique as described earlier (Yadav et al. 2014c). Epiphytic and endophytic bacteria were isolated using methods described by Holland and Polacco (1994) and Conn and Franco (2004), respectively. The imprint method as described by Holland et al. (2000) was also used to isolate epiphytic bacteria. The plates were incubated at 5–10 °C and populations were counted after 10–

15 days. Colonies that appeared were purified by repeated streaking on their respective medium plates. Pure cultures were maintained at 4 °C as slant and glycerol stock (25 %) at −80 °C for further use. All isolates were screened for tolerance to temperature (4–37 °C), salt concentration (5–20 % NaCl) and pH (3–11) according to methods described earlier (Yadav et al. 2014c).

PCR amplification of 16S rRNA gene and amplified rDNA restriction analysis

Genomic DNA was extracted by the method described by Kumar et al. (2013). The amount of DNA extracted was assessed by electrophoresis on an 0.8 % agarose gel. The 16S rRNA gene was amplified as described earlier (Pandey et al. 2013) using the universal primers pA (5′-AGAGTTTG ATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCA GCCGCA-3′) (Edwards et al. 1989). The PCR-amplified 16S rRNA gene was purified by a QIA quick PCR product purification kit (Qiagen, Hilden, Germany). Purified PCR products (100 ng) were digested separately with three restriction endonucleases *Alu*I, *Hae*III and *Msp*I (GeNei) in a 25-μL reaction volume, using the manufacturer's recommended buffer and temperature. The digested product together with a marker (100 bp, Bangalore GeNei, Bangalore, India) were resolved by gel electrophoresis (60 V cm^{-1}) in 2.5 % agarose gels in 1X TAE buffer containing 10 μg mL^{-1} ethidium bromide (EB). The gels were photographed by gel documentation system (Bio-Rad, Hercules, CA). Strong, clear bands were scored for similarity, and clustering analysis was undertaken using the software NTSYS-2.02e package (Exeter Software, East Setauket, NY). Similarity among the isolates was calculated by Jaccard's coefficient (Jaccard 1912) and a dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA) method.

16S rRNA gene sequencing and phylogenetic analysis

A representative strain from each amplified rDNA restriction analysis (ARDRA) group was selected for phylogenetic analysis. PCR products of partial 16S rRNA genes were sequenced with fluorescent terminators (Big Dye, Applied Biosystems, Foster City, CA) and run in a 3130xl Applied Biosystems ABI prism automated DNA sequencer at SCI Genome (Chennai, India). The 16S rRNA gene sequences were analysed using codon code aligner v.4.0.4 (http://www.codoncode.net). The sequences were aligned to those of closely related bacterial species available at the GenBank database using the BLASTn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed from the aligned datasets using the neighbour-joining (NJ) method (Saitou and Nei 1987) implemented in the program MEGA 4.0.2 (Tamura et al. 2007).

Accession numbers

The partial 16S rDNA sequences were submitted to NCBI GenBank and assigned accession numbers KF054746–KF054786 and KF572994–KF572997. All representative bacterial strains were deposited at the National Bureau of Agriculturally Important Microorganisms (NBAIM) culture collection facility, Mau Nath Bhanjan, Uttar Pradesh, India.

Statistical analysis

To compare bacterial diversity within the five sampling sites, the 16S rRNA gene sequences of isolates showing ≥ 97 % sequence similarity were grouped into the same cluster. The software Shannon Wiener Diversity Index/Shannon Entropy Calculator and Rarefaction Calculator was used to calculate the Shannon index (H), Evenness (J) and Simpson's index (D). Using 16S rRNA gene sequences, a rarefaction curve was generated to compare the relative diversity and coverage of each sample. Principal coordinate analysis (PCA) was used to determine the statistical correlation between population diversity of five sites surveyed (Rico et al. 2004). PCA was also performed for different physico-chemical properties and sampling sites, using the XLSTAT program (http://www.xlstat.com).

Plant growth promoting attributes

The functional diversity amongst recovered isolates was studied by qualitative screening of their plant growth promoting (PGP) attributes. Representative isolates from each cluster were initially screened qualitatively for PGP attributes. The production of gibberellic acid (GA), siderophore, hydrogen cyanide (HCN), indole 3-acetic acids (IAA) and ammonia were assessed by methods described by Brown and Burlingham (1968), Schwyn and Neilands (1987), Bakker and Schippers (1987), Bric et al. (1991) and Cappucino and Sherman (1992) respectively. Solubilisation of phosphorus, potassium and zinc were assessed according to the procedure of Mehta and Nautiyal (2001), Hu et al. (2006) and Saravanan et al. (2007) using Pikovskaya agar, Aleksandrov medium and nutrient agar medium containing 0.1 % insoluble zinc compounds [ZnO, ZnS, $Zn_3(PO_4)_2$ and $ZnCO_3$], respectively. The ability to fix nitrogen was evaluated using semi-solid nitrogen-free NFb medium (Boddey et al. 1995). The bacterial strains were screened for their ability to utilize 1-aminocyclopropane-1-carboxylate (ACC) as sole nitrogen source—a trait that is a consequence of the activity of the enzyme ACC deaminase (Jacobson et al. 1994). The bacterial strains were spotted on MDF (modified Dworkin and Foster medium) agar, i.e., MDF agar plates supplemented with 0.3 g L^{-1} ACC and MDF agar plates

with ammonium sulphate 0.3 g L^{-1}. After 72 h of incubation, plates were observed for growth. All assays were performed in triplicate at 10, 20 and 30 °C.

Quantitative estimation

P-solubilisation

Quantitative estimation of P-solubilisation was performed at three different incubation temperatures, 10, 20 and 30 °C, by inoculating 1 mL bacterial suspension in 25 mL National Botanical Research Institute phosphate medium broth (Mehta and Nautiyal 2001) containing 0.5 % tricalcium phosphate as sole phosphorus source in Erlenmeyer flasks (100 mL), and incubating for 7 days. At the end of the incubation the culture suspension was centrifuged at 8000 *g* for 15 min and the P content in the supernatant was estimated according to the method of Murphy and Riley (1962).

K-solubilisation

Quantitative estimation of K-solubilization was performed at three different incubation temperatures (10, 20 and 30 °C) by inoculating 1 mL bacterial suspension in 50 mL Aleksandrov medium in Erlenmeyer flasks (100 mL), and incubating for 7 days. The pH value was measured with a pH meter at intervals of 12 h. The cultures were diluted 1:1 (v:v) using 0.1 mol L^{-1} HCl, and harvested by centrifugation at 10,000 *g* for 15 min. The K content in the supernatant was estimated according to method of Monib et al. (1984) using atomic absorption spectrometry.

IAA production

Indole acetic acid production was estimated by inoculating the bacterial suspension of 1 mL aliquots (3×10^7 CFU mL^{-1}) in 25 mL Luria Bertani (LB) broth containing L-tryptophan (100 μg mL^{-1}) and incubating it at 10, 20 and 30 °C for 72 h on a rotary shaker at 180 rpm. The bacterial cultures were harvested and centrifuged at 8,000 *g* for 30 min. The supernatant (2 mL) was mixed with two to three drops of orthophosphoric acid and 4 mL Salkowski reagent (50 mL, 35 % sulphuric acid, 1 mL 0.5 M $FeCl_3$) and incubated for 25 min at room temperature. The development of pink-colour in the culture filtrate indicated the occurrence of IAA. Intensity of the colour was measured by spectrophotometer at 530 nm and the concentration of IAA produced by cultures was measured using a standard graph of IAA (Sigma-Aldrich, St. Louis, MO) obtained in the range of 10–100 μg mL^{-1} (Patten and Glick 2002).

Nitrogen fixation

The nitrogen-fixing attributes of the bacterial strains was tested using the acetylene reduction assay (ARA) described by Han and New (1998). Bacterial strains were inoculated into 12-mL vials containing 6 mL semisolid NFb medium and incubated for 24–36 h at 10, 20 and 30 °C. The vials were sealed with rubber septa and the gas phase of each vial was replaced with a gas mixture of nitrogen, air, and acetylene (90:10:10, v/v), and cultures were re-incubated at 10, 20 and 30 °C for 24 h. The amount of ethylene produced by acetylene reduction was measured by a Perkin Elmer F-11 gas chromatograph. The protein concentration of each strain was determined by the standard Bradford (1976) method.

Screening for biocontrol properties using plate assays

The in vitro antagonistic activity of the bacterial isolates was evaluated against three fungal pathogens (*Fusarium graminearum*, *Rhizoctonia solani* and *Macrophomina phaseolina*) according to the method described by Sijam and Dikin (2005). The fungal pathogens, which are involved in root-rot complex in crops, were obtained from the well characterized culture stock maintained at the Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India. Representative bacterial isolates were spotted on respective medium plates and incubated at 30 °C. Control plates with only the mycelial plug were set up and, when the pathogen had grown across these control plates, the diameter of growth in the challenge plates was measured. Triple culture assays were repeated three times per isolate for each fungus.

Results

Enumeration of wheat-associated bacteria

The population of bacteria in different samples collected from five sites of the NHZ of India with cold climatic conditions was enumerated (Table 1). The abundance of bacteria in the rhizospheric samples varied from 6.44×10^6 to 4.0×10^4 CFU g^{-1} soil, with the highest values found in samples from Uttarakhand, followed by Himachal Pradesh (6.02×10^6 CFU g^{-1} soil) and the lowest values in Jammu and Kashmir, followed by West Bengal (6.0×10^4 CFU g^{-1} soil). Populations in epiphytic and putative endophytic samples ranged from 6.32×10^6 to 9.8×10^5 CFU g^{-1} leaves and 2.98×10^6 to 1.21×10^6 CFU g^{-1} root, respectively. Among the media used, tryptic soya agar supported the highest population of bacteria 6.44×10^6 CFU g^{-1} soil and 6.32×10^6 CFU g^{-1} leaves in rhizospheric and epiphytic samples, respectively. Jensen's agar supported the least bacterial growth (Table 1). The

Table 1 Total viable count of bacteria associated with wheat cultivated in different parts of the northern hills zone (NHZ) of India. *JK* Jammu and Kashmir, *HP* Himachal Pradesh, *UK* Uttarakhand, *SI* Sikkim, *WB* West Bengal, *NA* nutrient agar, *KB* King's B agar, *AMS* ammonium minerals salt, *TSA* tryptic soy agar, T_3A T_3 agar, R_2A R_2 agar, *JA* Jensen's agar, *SEA* soil extract agar, *MDM* modified Dobereiner medium, *YEMA* yeast extract mannitol agar

Site	Total viable count (CFU g^{-1}leaves/ soil/ root × 10^6) on different media													
	Epiphytic				Rhizospheric								Endophytic	
	NA	KB	AMS	TSA	NA	T_3A	KB	R_2A	JA	AMS	SEA	TSA	MDM	YEMA
JK	2.98	2.21	0.98	3.21	3.56	0.98	2.4	2.56	0.04	3.66	2.88	4.23	1.21	2.58
HP	3.42	2.51	1.24	5.44	5.12	0.92	3.3	3.36	0.06	4.12	3.24	6.02	1.25	2.21
UK	4.28	3.96	1.55	6.32	5.48	1.74	3.9	3.70	0.09	4.88	3.62	6.44	1.58	2.98
SI	3.11	2.32	1.25	4.85	4.56	1.36	3.2	2.96	0.07	3.99	3.45	5.52	1.29	2.26
WB	3.39	3.23	1.36	5.26	4.55	1.56	3.1	2.45	0.06	3.85	3.25	5.87	1.27	2.22

highest population and number of bacteria were isolated from the rhizospheric samples, whereas the lowest number of bacteria was recovered from putative endophytic samples. The diversity of morphotypes was highest at Uttarakhand (75) and lowest (31) at Sikkim (see Table 4 below).

Physiological characterization of bacteria

All 247 isolates were screened for tolerance to temperature, salinity and pH and the results are presented for 45 representative strains. 26 strains could grow between 4 °C and 30 °C indicating that they were psychrotolerant, while 19 strains could grow between 10 °C and 37 °C indicating that they are mesophilic. Bacterial isolates also exhibited tolerance to different NaCl concentrations varying from 3 % to 10 % (w/v). Out of 45 representatives, all were tolerant to 5 % NaCl, while 11 and 12 isolates could tolerate 7 % and 10 % NaCl, respectively. Isolates could grow in the pH range of 5–11 and most strains could grow in the range of pH 5–9. Out of 45 representatives, 35 and 38 strains could grow at pH 5 and pH 7, while only 13 strains were able to grow at pH 11 (Supplementary Table 2).

PCR amplification of 16S rRNA genes and ARDRA

PCR amplification of 16S rDNA followed by ARDRA with three restriction endonucleases was carried out to look for species variation among the morphotypes selected. The 16S rDNA amplicons were digested with restriction enzymes, which generated profiles having three to seven fragments ranging in size from 100 to 900 bp (Fig. 1a). A combined dendrogram was constructed for each sampling site to determine the percent similarity among the isolates (Fig. 1b). At a level of >75 % similarity, the isolates were grouped into clusters; the number of clusters ranged from 19 (for West Bengal) to 33 (for Uttarakhand). The total number of clusters was 121, summed up for all sites (Supplementary Table 2).

16S rRNA gene sequencing and phylogenetic analysis

A total of 121 strains selected based on the restriction pattern generated by the 16S rRNA gene was sequenced. The sequence data were analysed by BLAST and the nearest match from GenBank data reported. Sequences were deposited with GenBank. DNA sequencing and phylogenetic analysis revealed that all the isolates showed ≥99–100 % similarity with sequences within GenBank. The phylogenetic tree of 45 identified bacteria was constructed to determine their affiliations (Fig. 2a, b). Analysis of the 16S rRNA gene sequences revealed that 65 %, 26 %, 8 % and 1 % bacteria belong to four phyla, namely Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes, respectively, with 45 distinct species of 19 genera (Fig. 2a,b). The Proteobacteria were the most predominant phylum followed by Firmicutes. Generally, *Arthrobacter* from Actinobacteria, *Bacillus* from Firmicutes and *Pseudomonas* from Proteobacteria were the genera most frequently recovered (Tables 2, 3).

Statistical analysis

Based on a similarity index of >97 % at the 16S rRNA gene sequence, the 247 isolates from the phyllosphere, endophytic and rhizosphere of wheat, could be categorised into 19–33 clusters (Table 4). Shannon's diversity index was highest ($H'=3.40$) for Uttarakhand samples whereas samples from West Bengal had the lowest value ($H'=2.85$). These observations were supported by bacterial diversity parameters, such as Simpson's index, Chao-1, and Evenness (Table 4). Principal coordinate analysis (PCA) was used to investigate relationships between bacterial diversity (Shannon's diversity index).

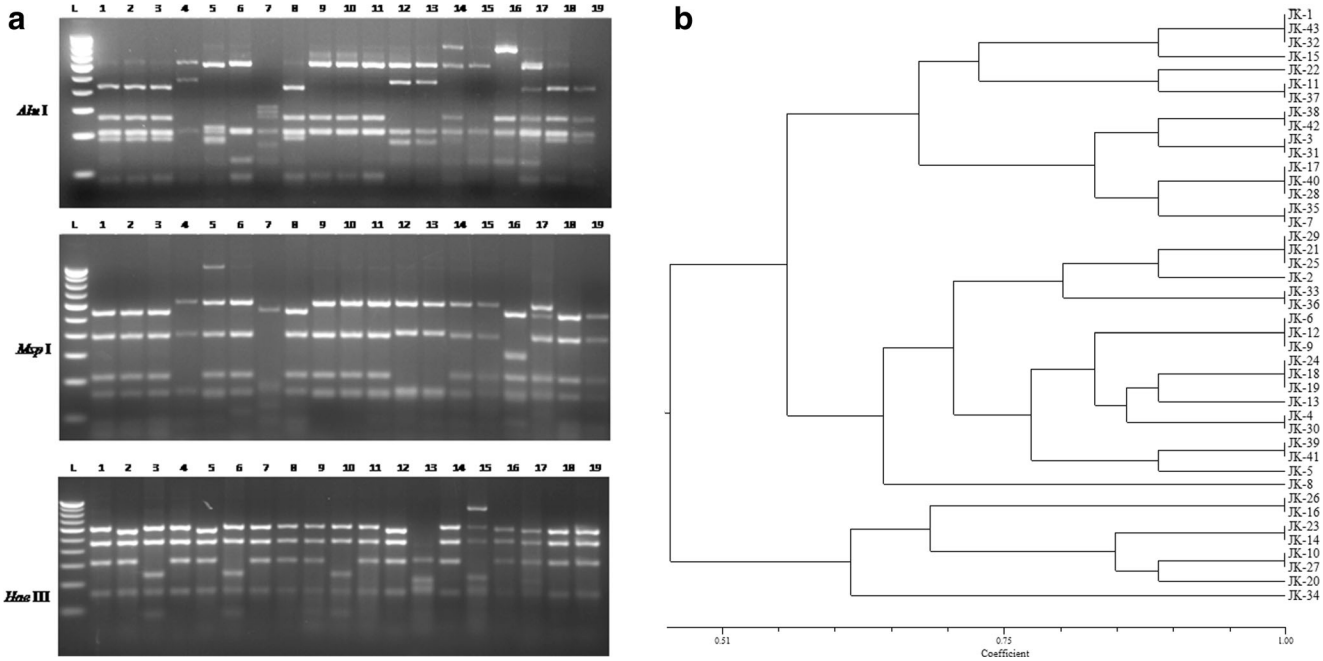


Fig. 1 **a** Representative restriction patterns of the amplified 16S rRNA gene of isolates generated using three restriction endonucleases: *Alu*I, *Msp*I and *Hae*III. **b** Representative dendrogram showing 23 clusters of 43 isolates from Jammu and Kashmir generated from the amplified rDNA restriction analysis (ARDRA) pattern of the 16S rDNA amplicon by three restriction endonucleases, *Alu*I, *Msp*I and *Hae*III, using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm and Jaccard's coefficient (Jaccard 1912)

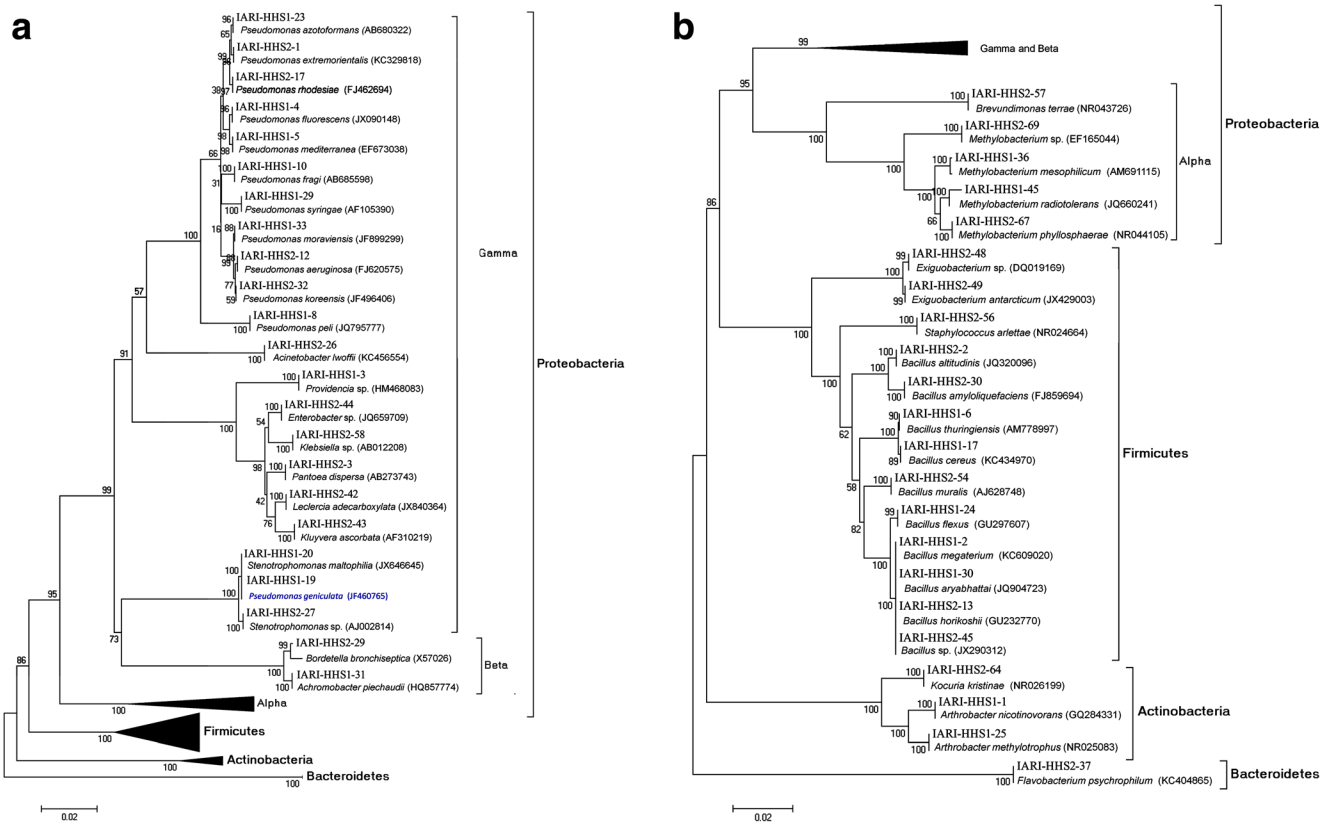


Fig. 2 **a**, **b** Phylogenetic trees showing the relationship among the 45 bacterial isolates according to 16S rRNA gene sequences with reference sequences obtained through BLAST analysis. The sequence alignment was performed using the CLUSTAL W program and trees were constructed using neighbour joining (NJ) with algorithm using MEGA4 software (Tamura et al. 2007)

Table 2 Identification and plant growth promoting attributes of bacterial isolates from wheat cultivating in different part of the NHZ of India: nearest phylogenetic relative, similarity, and solubilisation

Strain number	Nearest phylogenetic relative	Similarity (%)	Solubilisation		
			Phosphorus[a]	Potassium[a]	Zinc[a]
IARI-HHS1-1	*Arthrobacter nicotinovorans*	99	61.9±0.2	–	2.4±0.1
IARI-HHS1-25	*Arthrobacter methylotrophus*	99	55.9±1.4	–	3.3±0.2
IARI-HHS2-64	*Kocuria kristinae*	99	64.0±1.0	–	1.6±0.1
IARI-HHS2-37	*Flavobacterium psychrophilum*	99	66.0±0.7	–	2.0±0.1
IARI-HHS1-2	*Bacillus megaterium*	100	45.7±1.1	22±0.5	4.3±0.2
IARI-HHS1-6	*Bacillus thuringiensis*	100	63.2±1.4	–	3.1±0.1
IARI-HHS1-17	*Bacillus cereus*	99	–	–	–
IARI-HHS1-24	*Bacillus flexus*	98	47.8±0.1	–	–
IARI-HHS1-30	*Bacillus aryabhattai*	100	45.6±1.0	–	–
IARI-HHS2-2	*Bacillus altitudinis*	99	43.9±0.7	–	–
IARI-HHS2-13	*Bacillus horikoshii*	99	–	27±0.9	2.6±0.1
IARI-HHS2-30	*Bacillus amyloliquefaciens*	99	47.9±1.4	57±0.9	4.5±0.1
IARI-HHS2-45	*Bacillus* sp.	100	47.2±1.4	33±0.5	5.0±0.1
IARI-HHS2-48	*Exiguobacterium* sp.	100	43.7±0.9	–	1.1±0.1
IARI-HHS2-49	*Exiguobacterium antarcticum*	99	–	22±0.8	2.4±0.1
IARI-HHS2-54	*Bacillus muralis*	100	–	–	2.0±0.1
IARI-HHS2-56	*Staphylococcus arlettae*	99	–	–	1.5±0.1
IARI-HHS2-57	*Brevundimonas terrae*	99	–	–	–
IARI-HHS1-36	*Methylobacterium mesophilicum*	98	–	–	–
IARI-HHS1-45	*Methylobacterium radiotolerans*	100	–	–	–
IARI-HHS2-67	*Methylobacterium phyllosphaerae*	99	–	–	–
IARI-HHS2-69	*Methylobacterium* sp.	100	–	–	–
IARI-HHS1-31	*Achromobacter piechaudii*	100	–	33±1.2	1.6±0.1
IARI-HHS2-29	*Bordetella bronchiseptica*	99	48.6±0.9	–	2.6±0.1
IARI-HHS1-3	*Providencia* sp.	100	34.4±1.2	–	3.6±0.1
IARI-HHS1-4	*Pseudomonas fluorescens*	99	54.6±0.9	–	–
IARI-HHS1-5	*Pseudomonas mediterranea*	98	–	–	–
IARI-HHS1-8	*Pseudomonas peli*	100	51.6±1.0	–	2.1±0.1
IARI-HHS1-10	*Pseudomonas fragi*	98	–	–	–
IARI-HHS1-19	*Pseudomonas geniculata*	99	45.0±1.2	–	–
IARI-HHS1-20	*Stenotrophomonas maltophilia*	100	55.7±0.5	28±1.2	–
IARI-HHS1-23	*Pseudomonas azotoformans*	99	40.4±1.1	–	–
IARI-HHS1-29	*Pseudomonas syringae*	100	–	–	–
IARI-HHS1-33	*Pseudomonas moraviensis*	99	–	–	–
IARI-HHS2-1	*Pseudomonas extremorientalis*	100	57.7±0.6	–	2.6±0.1
IARI-HHS2-3	*Pantoea dispersa*	99	–	–	2.1±0.1
IARI-HHS2-12	*Pseudomonas aeruginosa*	100	–	–	2.2±0.2
IARI-HHS2-17	*Pseudomonas rhodesiae*	99	51.6±1.4	–	5.1±0.2
IARI-HHS2-26	*Acinetobacter lwoffii*	98	21.6±1.0	–	–
IARI-HHS2-27	*Stenotrophomonas* sp.	100	23.7±0.5	22±0.5	–
IARI-HHS2-32	*Pseudomonas koreensis*	99	25.0±1.2	–	–
IARI-HHS2-42	*Leclercia adecarboxylata*	99	–	–	–
IARI-HHS2-43	*Kluyvera ascorbata*	99	–	–	–
IARI-HHS2-44	*Enterobacter* sp.	100	–	–	–
IARI-HHS2-58	*Klebsiella* sp.	100	–	16±0.5	–

[a] Numerical values are mean±SD of three independent observations; phosphorus (mg L^{-1}); potassium (mg mL^{-1}). Radius of halo zone in millimetres for siderophore and zinc solubilisation; − negative for the attribute; + positive for the attribute

Table 3 Identification and plant growth promoting attributes of bacterial isolates from wheat cultivating in different parts of the NHZ of India: production and other activities. *IAA* Indole 3-acetic acid, *GA* gibberellic acid, *HCN* hydrogen cyanide, *ACC* 1-aminocyclopropane-1-carboxylate, *N_2F* N_2fixation

Strain number	Production					Other activities		
	IAA[a]	Siderophore[a]	GA	HCN	NH_3	ACC	N_2F[a]	Biocontrol
IARI-HHS1-1	27.8±1.2	1.0±0.1	−	−	+	−	18.25±1.2	−
IARI-HHS1-25	21.4±1.3	4.9±0.1	−	−	+	+	9.65±1.5	+
IARI-HHS2-64	20.4±1.1	2.1±0.1	+	+	−	−	−	−
IARI-HHS2-37	11.4±1.5	2.6±0.1	−	+	+	+	−	−
IARI-HHS1-2	16.6±1.0	3.5±0.2	−	−	+	−	−	−
IARI-HHS1-6	−	2.5±0.1	−	+	+	−	−	−
IARI-HHS1-17	−	2.6±0.1	−	−		−	−	−
IARI-HHS1-24	−	−	−	+	+	−	48.36±1.3	−
IARI-HHS1-30	15.6±0.7	−	+	+	−	−	84.21±1.2	−
IARI-HHS2-2	6.6±1.0	−	+	+	−	−	79.26±1.4	+
IARI-HHS2-13	8.75±1.2	1.8±0.2	−	+	−	+	−	+
IARI-HHS2-30	17.8±1.2	2.1±0.1	+	+	−	−	56.36±1.5	+
IARI-HHS2-45	−	1.5±0.1	−	+	−	−	22.69±1.2	+
IARI-HHS2-48	−	1.2±0.2	−	−	+	−	−	+
IARI-HHS2-49	22.8±0.8	3.1±0.1	−	+	−	−	−	+
IARI-HHS2-54	28.6±1.0	4.1±0.2	−	+	+	−	−	−
IARI-HHS2-56	−	3.2±0.2	−	−	−	−	−	+
IARI-HHS2-57	17.2±1.1	2.2±0.5	+	−	+	−	−	+
IARI-HHS1-36	12.2±1.0	3.2±0.5	+	−	+	+	−	−
IARI-HHS1-45	18.2±1.0	4.2±0.5	+	−	+	+	−	−
IARI-HHS2-67	27.2±1.0	2.4±1.2	+	−	+	+	−	−
IARI-HHS2-69	−	2.2±1.0	+	−	+	+	−	−
IARI-HHS1-31	−	3.1±0.1	−	+	+	−	−	+
IARI-HHS2-29	15.2±1.1	1.6±0.2	−	+	−	−	11.99±1.2	+
IARI-HHS1-3	70.8±1.5	4.1±0.1	+	+	+	+	23.25±1.6	−
IARI-HHS1-4	20.8±1.5	1.8±0.1	−	−	+	+	20.25±1.3	+
IARI-HHS1-5	25.4±1.2	−	−	+	+	−	58.85±1.5	+
IARI-HHS1-8	69.1±0.5	4.6±0.1	−	−	−	−	66.55±1.2	−
IARI-HHS1-10	18.2±1.1	4.4±0.1	−	−	−	+	−	+
IARI-HHS1-19	66.7±0.5	2.5±0.2	−	+	−	−	78.20±1.6	−
IARI-HHS1-20	66.1±0.7	2.4±0.1	+	+	+	+	+	+
IARI-HHS1-23	15.1±0.4	3.4±0.1	+	+	+	+	98.75±1.8	+
IARI-HHS1-29	+	2.2±0.2	+	+	+	+	36.15±1.3	+
IARI-HHS1-33	27.8±1.2	2.1±0.2	+	+	+	+	+	+
IARI-HHS2-1	12.2±1.1	5.1±0.2	+	+	+	+	+	+
IARI-HHS2-3	+	4.6±0.1	+	+	+	+	+	+
IARI-HHS2-12	+	+	+	+	+	+	+	+
IARI-HHS2-17	+	2.6±0.1	+	+	+	+	+	+
IARI-HHS2-26	15±0.4	+	+	+	+	+	10.23±1.2	+
IARI-HHS2-27	36.1±0.7	+	+	+	+	+	13.28±1.6	+
IARI-HHS2-32	36.7±0.5	3.5±0.2	+	+	+	+	+	+
IARI-HHS2-42		+	+	+	+	+	+	+
IARI-HHS2-43	35.5±1.2	+	+	+	+	+	+	+
IARI-HHS2-44	22.4±1.0	+	+	+	+	+	+	+
IARI-HHS2-58	24.2±1.0	2.8±0.1	+	+	+	+	+	+

[a] Numerical values are mean±SD of three independent observations; indole acetic acid (IAA; μg mg^{-1} protein day^{-1}); N_2fixation (nmol ethylene h^{-1} mg^{-1} protein)

Table 4 Diversity indices for the isolates from wheat growing at five different sites in the NHZ of India

	Jammu and Kashmir	Himachal Pradesh	Uttarakhand	Sikkim	West Bengal
No. of isolates	43	55	75	31	43
Species richness	23	25	33	21	19
Evenness (J′)	0.92	0.92	0.90	0.93	0.90
Shannon (H)	3.05	3.13	3.40	2.97	2.85
Simpson's (D)	0.94	0.95	0.96	0.94	0.93
Chao-1	25.55	25.77	35.15	28.33	19.6
Niche specific bacteria	*Pseudomonas peli*, *P. mediterranea*, *P. geniculata*, *Bacillus muralis*	*P. fragi*, *P. rhodesiae*, *A. methylotrophus*	*Providencia* sp., *P. koreensis*, *P. azotoformans*, *L. adecarboxylata*	*Klebsiella* sp., *P. syringae*, *P. extremorientalis*	*B. horikoshii*, *Achromobacter piechaudii*

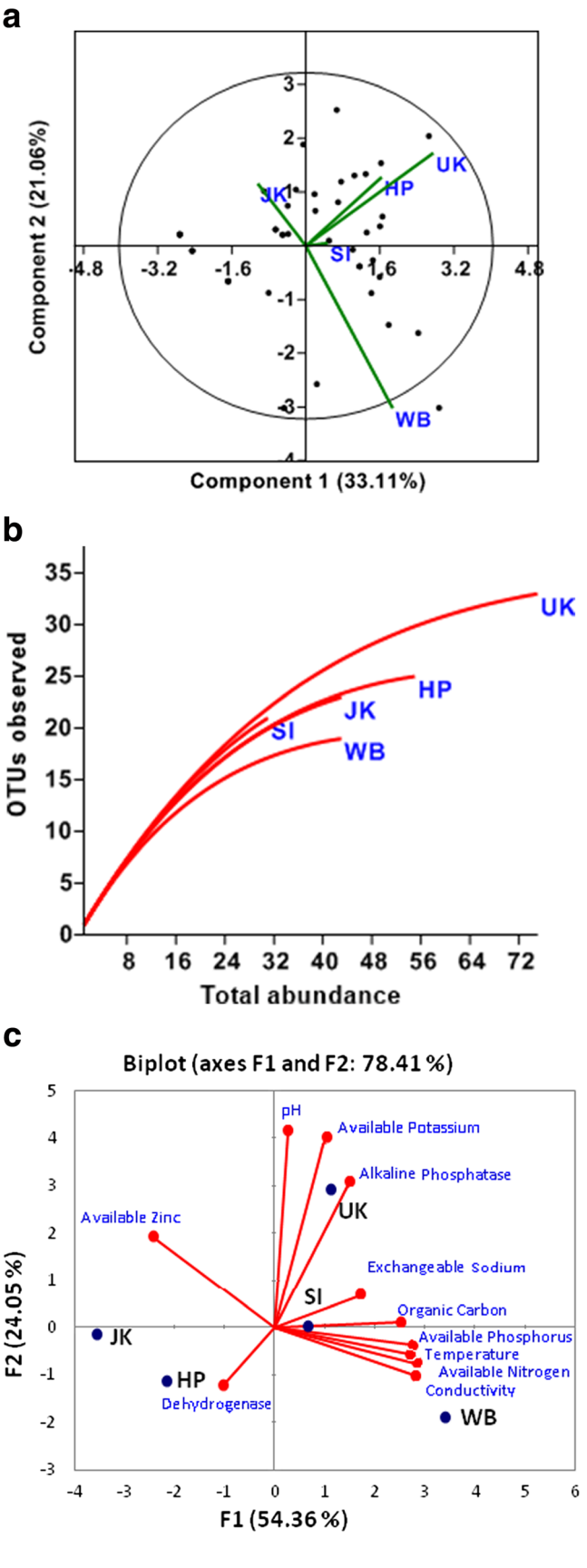
The first two dimensions of PCA (PCA1 and PCA2) explained 52.17 % of the total variation, with component 1 accounting for 33.11 % and component 2 for 21.06 % of the variance (Fig. 3a). The 16S rRNA gene sequencing results revealed that *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus flexus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Methylobacterium phyllosphaerae*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* were the common strains recovered from all five sites surveyed, followed by *Pantoea dispersa*, *Kluyvera ascorbata*, *Staphylococcus arlettae* and *Methylobacterium mesophilicum*, which shared four out of the five sites. In addition to the many common microorganisms, the different sites also had unique culturable bacterial species. Various microbes were isolated exclusively from the samples collected from particular sites, exhibiting the niche-specific presence of bacteria (Table 4). The individual rarefaction curves for the samples of all five sites indicated that the bacterial population was the least diverse in West Bengal followed by Sikkim, and most diverse in Uttarakhand followed by Himachal Pradesh (Fig. 3b). Correlation analysis proved the existence of a significant relationship between the different parameters and sampling sites. The first two factorial axes of the biplot represented 54.36 % to 78.41 % variance in the data (Fig. 3c).

Plant growth promoting attributes

The representative strains were screened for plant growth promoting (PGP) traits. Out of 45 representatives, 24, 21 and 9 strains exhibited solubilisation of phosphorus, zinc and potassium, respectively (Tables 2, 3). Of the 45 strains, 24 produced ammonia and 36 strains IAA, while only 13 strains produced GA (Tables 2, 3). ACC deaminase activity was shown by 15 strains, while 17 isolates showed N_2-fixation. N_2-fixation activity varied from 9.65±1.5 to 98.75±1.8 nmol ethylene h^{-1} mg^{-1} protein (Tables 2, 3) Among PGP activities, siderophore-producing strains were highest (15 %) when compared to IAA production (14 %), P-solubilisation (11 %), ammonia production (11 %), zinc solubilisation (9 %), HCN production (9 %), nitrogen fixation (8 %), ACC deaminase (7 %) biocontrol (6 %), GA production (6 %) and K-solubilisation (5 %). The strain IARI-HHS2-37 solubilised the highest amount of phosphorus (66.0±0.7 mg L^{-1}) followed by IARI-HHS2-64 (64.0±1.0 mg L^{-1}). Strain IARI-HHS1-3 showed highest IAA production (70.8±1.5 μg mg^{-1} protein day^{-1}) followed by IARI-HHS1-8 (69.1±0.5 μg mg^{-1} protein day^{-1}). Highest solubilisation of potassium was shown by strain IARI-HHS2-30 (57±0.9 mg L^{-1}) while strain IARI-HHS2-17 showed highest zinc solubilisation (5.1±0.2 mm). Strain IARI-HHS1-23 exhibited the highest nitrogen fixation activity (98.75±1.8 nmol ethylene h^{-1} mg^{-1} protein) followed by strain IARI-HHS1-30 (84.21±1.2 nmol ethylene h^{-1} mg^{-1} protein) (Tables 2, 3). Fourteen strains showed antagonistic activity against *Fusarium graminearum*, *Rhizoctonia solani* and *Macrophomina phaseolina* (Table 2, 3). Among the 45 strains, 12 identified as *Arthrobacter methylotrophus*, *Arthrobacter nicotinovorans*, *Bacillus altitudinis*, *Bacillus amyloliquefaciens*, *Bacillus horikoshii*, *Bacillus megaterium*, *Bacillus* sp., *Bordetella bronchiseptica*, *Exiguobacterium antarcticum*, *Flavobacterium psychrophilum*, *Kocuria kristinae* and *Providencia* sp., exhibited more than six different PGP activities at low temperature. PGP traits of phosphate solubilisation, siderophore production, IAA production and ACC deaminase activity are represented in a Venn diagram in Fig. 4.

Discussion

Extreme low temperature environments harbour microbial diversity, which may be fundamental to the maintenance and conservation of global genetic resources. Our present study deciphers the diversity of microbial communities coupled with the epiphytic, putative endophytic and rhizosphere of wheat crops, and investigated their PGP attributes. A total of 247 wheat-allied bacterial isolates was obtained from different



◀ **Fig. 3** **a** Principal coordinate analysis (PCA) of the diversity indices (H) of the16S rRNA PCR-ARDRA profiles of the five sites in relation to 16S rRNA gene sequences, Component 1 and component 2 accounted for 33.11 % and for 21.06 % of the total variation, respectively. **b** Rarefaction curves of observed operational taxonomic units (OTUs) in the five sites from the NHZ of India. **c** Biplot showing relationship between different sampling sites and temperature, pH, conductivity, organic carbon, available NPK and zinc, exchangeable sodium, dehydrogenase and alkaline phosphatase. *JK* Jammu and Kashmir, *HP* Himachal Pradesh, *UK* Uttarakhand, *SI* Sikkim, *WB* West Bengal

sites in the NHZ of India. To the best of our knowledge, this is first report to elucidate the bacterial diversity associated with epiphytic, endophytic and rhizosphere of wheat crops with their PGP attributes at low temperature conditions.

Sequencing of the 16S rRNA gene of the representative strains from each cluster of all five different sites resulted in their identification and the 45 distinct species selected were subjected to phylogenetic analysis. Partial sequencing of the smaller subunit of the 16S rRNA gene assigned and grouped all the representative bacteria into four phyla: Actinobacteria (8 %), Bacteroidetes (1 %), Firmicutes (26 %) and Proteobacteria (65 %) (Fig. 5a). Among the five sites analysed, the highest diversity was found in Jammu and Kashmir and Uttarakhand, with all four phyla being represented at these sites (Fig. 5b). Many species of *Bacillus*, *Pseudomonas* and *Methylobacterium* were found to be common to all five different sites. Apart from dominant and common species of bacteria at different sites, niche-specific bacterial species were also identified at all the sites. These niche-specific bacteria were represented by *Pseudomonas peli*,

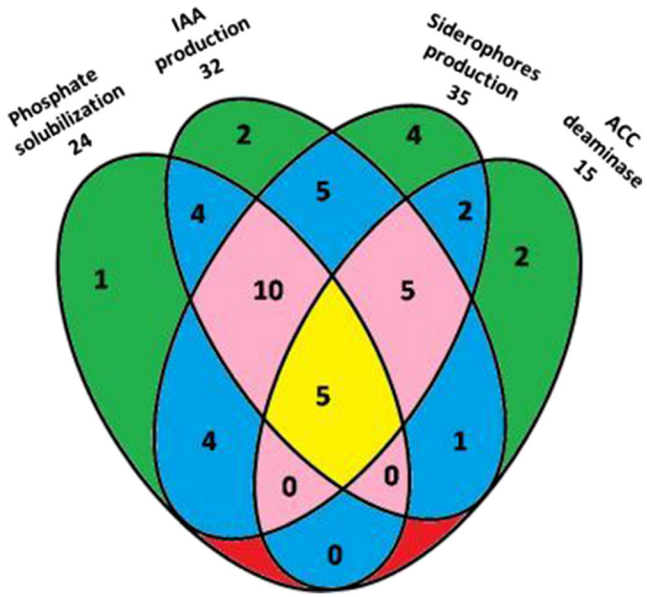


Fig. 4 Venn diagram illustrating the number of plant growth promoting bacteria (PGPB) showing the PGP traits of phosphate solubilisation, ACC deaminase, siderophore production and indole production

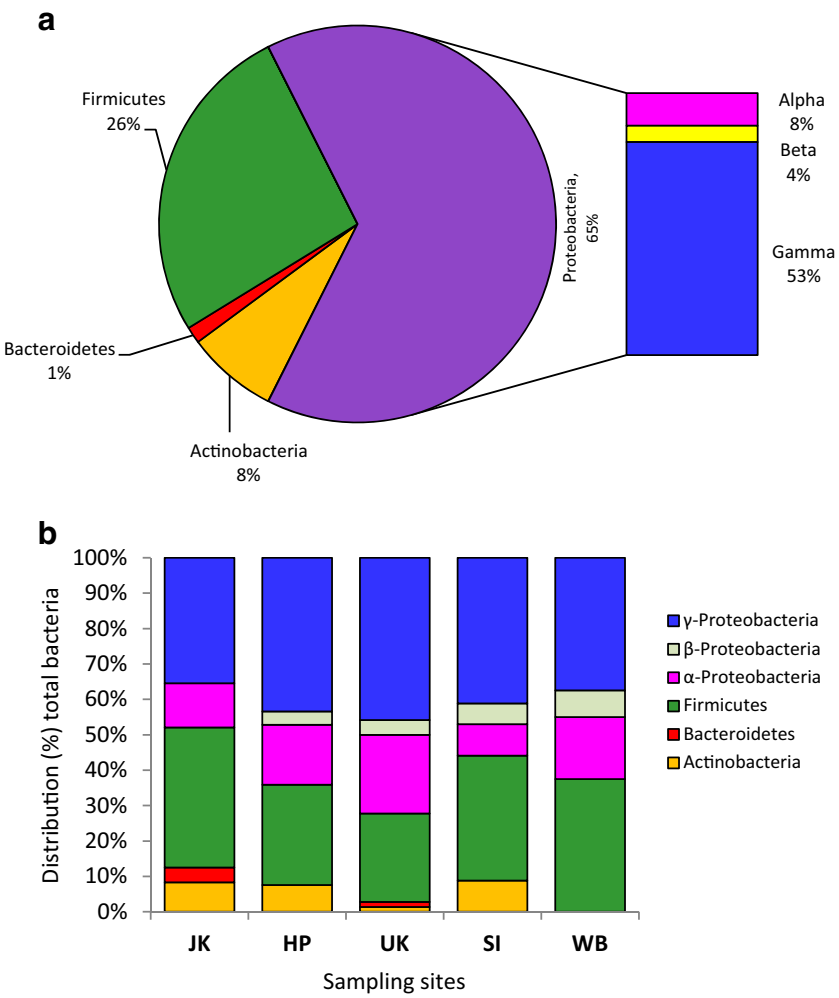


Fig. 5 a,b Abundance of different bacteria. **a** Distribution of phyla and groups in the samples surveyed. **b** Distribution of total bacteria in five sampling sites. *JK* Jammu and Kashmir, *HP* Himachal Pradesh, *UK* Uttarakhand, *SI* Sikkim, *WB* West Bengal

P. mediterranea, *P. geniculata* and *Bacillus muralis* at Jammu and Kashmir; *Pseudomonas fragi*, *P. rhodesiae* and *Arthrobacter methylotrophus* at Himachal Pradesh; *Pseudomonas azotoformans*, *Pseudomonas koreensis*, *Leclercia adecarboxylata* and *Providencia* sp. at Uttarakhand; *Pseudomonas syringae*, *Pseudomonas extremorientalis* and *Klebsiella* sp. at Sikkim; *Achromobacter piechaudii* and *Bacillus horikoshii* at West Bengal (Supplementary Table 1; Table 4). Other reports are also available on niche-specific bacterial diversity from different habitats (Kumar et al. 2014; Verma et al. 2014; Yadav et al. 2014a–c;).

In our study, Proteobacteria were most dominant phylum followed by Firmicutes. Of 45 representative strains, 5 distinct species with two genera *Brevundimonas* and *Methylobacterium* belonged to Alphaproteobacteria, while two strains *Achromobacter piechaudii* and *Bordetella bronchiseptica* belonged to Betaproteobacteria. The most predominant class of Proteobacteria was Gammaproteobacteria, consisting of 21 distinct species distributed into three orders: Pseudomonadales with 12 strains *Acinetobacter lwoffii*, *Pseudomonas aeruginosa*, *Pseudomonas azotoformans*, *Pseudomonas extremorientalis*, *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Pseudomonas koreensis*, *Pseudomonas mediterranea*, *Pseudomonas moraviensis*, *Pseudomonas peli*, *Pseudomonas rhodesiae* and *Pseudomonas syringae*; Enterobacteriales with six strains *Enterobacter* sp., *Klebsiella* sp., *Kluyvera ascorbata*, *Leclercia adecarboxylata*, *Pantoea dispersa* and *Providencia* sp.; Xanthomonadales with three strains *Pseudomonas geniculata*, *Stenotrophomonas* sp. and *Stenotrophomonas maltophilia*. *Pseudomonas geniculata* was included in the cluster of the genus *Stenotrophomonas* and the same prediction was reported by Anzai et al. (2000) (Fig. 2a).

The second dominant phylum is Firmicutes, including three families; Bacillaceae with ten strains *Bacillus altitudinis*, *Bacillus amyloliquefaciens*, *Bacillus aryabhattai*, *Bacillus cereus*, *Bacillus flexus*, *Bacillus horikoshii*, *Bacillus megaterium*, *Bacillus muralis*, *Bacillus* sp. and *Bacillus thuringiensis*; Bacillales Incertae Sedis with two strains *Exiguobacterium antarcticum* and *Exiguobacterium* sp.; Staphylococcaceae with one strain *Staphylococcus arlettae*.

Three strains belonged to the phylum Actinobacteria, represented by *Arthrobacter nicotinovorans*, *A. methylotrophus* and *Kocuria kristinae*; and one strain, *Flavobacterium psychrophilum* belonged to the phylum Bacteroidetes (Fig. 2b).

In the present investigation, different groups of bacteria, such as *Providencia*, *Pseudomonas*, *Arthrobacter*, *Achromobacter*, *Brevundimonas* and *Methylobacterium*, were isolated from wheat. The epiphytic bacterial strains were identified as *Arthrobacter methylotrophus*, *Pseudomonas rhodesiae*, *Methylobacterium mesophilicum*, *Methylobacterium phyllosphaerae*, *Methylobacterium radiotolerans* and *Methylobacterium* sp., in which pink-pigmented facultative methylotrophs (PPFMs) were most dominant. PPFMs are reported to influence the seed germination and growth of many crop plants (Madhaiyan and Poonguzhali 2014; Verma et al. 2014). *Arthrobacter methylotrophus* and *Pseudomonas rhodesiae* were identified as epiphytic PGPB for the first time herein, from wheat growing at high altitude and low temperature environments. Hantsis-Zacharov and Halpern (2007) first reported *Arthrobacter methylotrophus* from raw milk. *Arthrobacter methylotrophus* is a niche-specific bacterium isolated from wheat growing in Shimla, Himachal Pradesh. It is a yellow-coloured psychrotolerant bacterium that can tolerate 5 % NaCl and produces IAA, siderophore and ammonia; solubilise phosphorus and zinc; fix nitrogen; exhibits ACC deaminase activity and is antagonistic against *Fusarium graminearum*, *Rhizoctonia solani* and *Macrophomina phaseolina* at low temperatures. *Pseudomonas rhodesiae* was reported earlier from natural mineral waters (Coroler et al. 1996). It is an orange-coloured psychrotolerant bacterium that can tolerate 5 % NaCl and produces siderophore, as well as solubilising phosphorus and zinc at low temperatures.

Among PGPB, members of *Bacillus* and *Bacillus* derived genera (BBDG) are ubiquitous bacteria that include both free living PGPB and pathogenic species. PGPB belonging to BBDG have been reported to enhance the growth of several plants such as wheat (Beneduzi et al. 2008), oilseed rape (Ghosh et al. 2003), tomato (Kloepper et al. 1980), sugar beet (Bargabus et al. 2002), sorghum (Zinniel et al. 2002) and peanut (Dey et al. 2004). *Bacillus thuringiensis* is currently used in the biological control of insects in crop protection. Apart from BBDG, another group of PGPB belong to the genus *Pseudomonas* and in the present study isolated species of *Pseudomonas* identified as PGPB were *Pseudomonas fluorescens*, *P. mediterranea*, *P. peli*, *P. fragi*, *P. geniculata*, *P. azotoformans*, *P. syringae P. moraviensis*, *P. extremorientalis*, *P. aeruginosa*, *P. rhodesiae* and *P. koreensis*. It was reported earlier that *Pseudomonas* PGPB are highly resistant to various environmental stresses (Vyas et al. 2009).

In the present investigation, *Acinetobacter lwoffii*, *Bacillus amyloliquefaciens*, *Klebsiella* sp., *Pantoea dispersa*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* were isolated and identified from the endophytic sphere of wheat. *Acinetobacter lwoffii*, *Bacillus amyloliquefaciens* and *Pantoea dispersa* were isolated for the first time from internal tissues of wheat. These species have been isolated previously from different crops as endophytic bacteria. For example, *Acinetobacter lwoffii* was isolated from internal shoot tips of banana (Thomas and Soly 2009). It is a cream-/white-coloured psychrotolerant and alkalitolerant bacterium that can tolerate 10 % NaCl and produces IAA and HCN; it also solubilises phosphorus and exhibits nitrogen fixation. *Bacillus amyloliquefaciens* was reported as endophytic from *Scutellaria baicalensis* (Sun et al. 2006). It is ubiquitous, isolated from all five study sites, forms yellow-coloured colonies on nutrient agar medium, grows in the pH range of 5–11, tolerates 10 % NaCl, produces IAA, gibberllic acid, HCN, siderophore; solubilises phosphorus, potassium, zinc; exhibits biocontrol and nitrogen fixation activity. *Pantoea dispersa* was reported earlier from seeds of maize (*Zea mays* L.) (Liu et al. 2013). It is an orange-coloured mesophilic bacterium that can tolerate 5 % NaCl, produces siderophore, and can solubilise zinc. Endophytic bacteria have the potential to exert several beneficial effects on host plants, such as stimulation of plant growth, nitrogen fixation and induction of resistance to plant pathogens (Jha and Kumar 2009; Verma et al. 2014).

There were obvious differences among the epiphytic, putative endophytic and rhizosphere bacterial communities in term of CFU count and phylotypes. Lower bacterial diversity was observed in the putative endophytes compared to epiphytic and rhizospheric. The PPFMs *Methylobacterium mesophilicum*, *Methylobacterium radiotolerans*, *Methylobacterium phyllosphaerae* and *Methylobacterium* sp. were identified as epiphytic. In our present study, *Brevundimonas* and *Methylobacterium* were isolated as epiphytic whereas *Acinetobacter lwoffii*, *Bacillus amyloliquefaciens*, *Pantoea dispersa* and *Klebsiella* sp. were found as putative endophytic only. *Stenotrophomonas maltophilia* was recovered from both putative endophytic and rhizospheric communities. *Providencia* sp., *Pseudomonas fluorescens*, *Pseudomonas geniculata*, *Pseudomonas azotoformans*, *Arthrobacter methylotrophus*, *Achromobacter piechaudii*, *Pseudomonas extremorientalis*, *Pseudomonas rhodesiae* and *Staphylococcus arlettae* were isolated from epiphytic and rhizospheric samples.

Wheat-associated PGPB have a high potential for agriculture because they can improve plant growth under limiting or stress conditions of temperatures (Mishra et al. 2011; Selvakumar et al. 2011). Psychrotrophic PGPB were recently used to improve cold stress in plants (Selvakumar et al. 2011; Bisht et al. 2013). PGPB can facilitate the proliferation of their plant host directly through the production of stimulatory

phytohormones. The auxin IAA is an important phytohormone produced by PGPB, and treatment with auxin-producing rhizobacteria has been shown to increase plant growth. IAA—among the best characterized auxins—is a phytohormone essential for the growth and development of plants. The capacity to synthesize IAA is widespread among soil- and plant-associated bacteria. It has been estimated that 80 % of bacteria isolated from the rhizosphere can produce this plant growth regulator. Along with phytohormone production, plant growth promotion is known to be mediated by a variety of mechanisms including solubilisation of phosphorus, potassium and zinc; production of ammonia, siderophores and HCN (Tilak et al. 2005; Bisht et al. 2013; Verma et al. 2014). The ACC deaminase activity of microbes can lower plant ethylene levels and in turn facilitate plant growth. The production of siderophores by microbes influences plant growth by binding to the available iron form (Fe^{3+}) in the rhizosphere and, by this process, iron is made unavailable to phytopathogens and thus the siderophore protects plant health. HCN production by bacteria has been reported as a major means of control of diseases of crop plants. This may be attributed to the presence of a cyanide-resistant respiratory pathway in plants (Jacobson et al. 1994; Mishra et al. 2011; Selvakumar et al. 2011; Verma et al. 2014).

Phosphate (P) and potassium (K) are major essential macronutrients for biological growth and development. However, the concentrations of soluble P and K in soil are usually very low, as the biggest proportions of P and K in soil are present in insoluble rocks, minerals and other deposits (Goldstein 1994). The most efficient phosphate solubilizing bacteria (PSB) belong to the genera *Bacillus* and *Pseudomonas* (Selvakumar et al. 2011; Yadav et al. 2014a, b). There are considerable populations of P- or K-solubilising bacteria in soil and in the plant rhizosphere. P-solubilising bacteria (PSB) have the ability to solubilise inorganic phosphate compounds, such as tricalcium phosphate (Vyas et al. 2009). Among PGP attributes, P-solubilisation at low temperature is probably the best characterised (Gulati et al. 2008; Mishra et al. 2011; Selvakumar et al. 2011; Yadav et al. 2014a, b). In the present study, P-solubilisation activity was exhibited by many genera such as *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Bordetella*, *Exiguobacterium*, *Flavobacterium*, *Kocuria*, *Providencia*, *Pseudomonas* and *Stenotrophomonas*. K-solubilising bacteria (KSB) have been found to resolve potassium, silicon and aluminium from insoluble minerals (Aleksandrov et al. 1967). BBDG were best characterized for K-solubilisation (Sheng 2005). To the best of our knowledge, *Achromobacter* and *Stenotrophomonas* are reported as K-solubilisers at low temperature for the first time. KSB may have a use in the amelioration of K-deficient soil in agriculture at low temperatures.

Zinc is a nutrient at low concentration but is toxic at higher concentration. The solubilisation of zinc might limit the growth of the bacteria at higher levels. Zinc solubilisation by bacteria has an immense importance to zinc nutrition to plants. Among BBDG, three strains—*Bacillus megaterium*, *Bacillus amyloliquefaciens* and *Bacillus* sp.—exhibited all three types of solubilisation: phosphorus, potassium and zinc. Bacteria that fix atmospheric nitrogen into biologically useable ammonium are known to affect, directly or indirectly, plant growth. In the present investigation, a diverse group of bacteria were characterized for nitrogen fixation, such as *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Bordetella*, *Providencia*, *Pseudomonas* and *Stenotrophomonas*. Selected nitrogen fixing, P- and K-solubilising bacteria could be used effectively as biofertilisers in place of chemical fertilizers. The resulting NPK could increase soil productivity to improve sustainability of agricultural production.

In conclusion, the potential utility of such cold-active bacterial strains in the context of hill and mountain agro-ecosystems is immense, considering the unique crops growing in the climatic conditions of high altitude agricultural systems. Such systems require situation-specific microbial inoculants that withstand extremities of cold and retain their functional traits for PGP. The PGP potential of the bacterial strains dealt with in this study requires further evaluation and validation before their use as bio-inoculants. The selection of native functional PGP microorganisms is a mandatory step for reducing the use of energy-intensive chemical fertilizers. The strains reported in this study seem to be ideal candidates for promotion as bio-inoculants, due to their cold tolerance and multiple PGP traits. PGP psychrotolerant bacteria represent another group of important isolates that could be developed as suitable inoculants for winter season crops grown in alpine, sub-alpine, high altitude and low temperature regions.

Acknowledgements The authors are grateful to the Division of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi and Department of Biotechnology (DBT), Ministry of Science and Technology for providing the facilities and financial support needed to undertake this investigation.

Declaration The experiments undertaken comply with the current laws of India, the country where the investigation was undertaken. There are no conflicts of interest.

References

Aleksandrov VG, Blagodyr RN, Ilev IP (1967) Liberation of phosphoric acid from apatite by silicate bacteria. Mikrobiol Z 29:111–114

Anzai Y, Kim H, Park JY, Wakabayashi H, Oyaizu H (2000) Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. Int J Syst Evol Microbiol 50:1563–1589

Araújo WL, Marcon J, Maccheroni W, van Elsas JD, van Vuurde JW, Azevedo JL (2002) Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Appl Environ Microbiol 68:4906–4914

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57:233–266

Bakker AW, Schippers B (1987) Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* SPP-mediated plant growth-stimulation. Soil Biol Biochem 19:451–457

Bargabus R, Zidack N, Sherwood J, Jacobsen B (2002) Characterisation of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. Physiol Mol Plant Pathol 61:289–298

Beneduzi A, Peres D, da Costa PB, Bodanese Zanettini MH, Passaglia LMP (2008) Genetic and phenotypic diversity of plant-growth-promoting bacilli isolated from wheat fields in southern Brazil. Res Microbiol 159:244–250

Bisht SC, Mishra PK, Joshi GK (2013) Genetic and functional diversity among root-associated psychrotrophic Pseudomonad's isolated from the Himalayan plants. Arch Microbiol 195:605–615

Boddey R, De Oliveira O, Urquiaga S, Reis V, De Olivares F, Baldani V, Döbereiner J (1995) Biological nitrogen fixation associated with sugar cane and rice: contributions and prospects for improvement. Plant Soil 174:195–209

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bray RH, Kurtz L (1945) Determination of total, organic, and available forms of phosphorus in soils. Soil Sci 59:39–46

Bric JM, Bostock RM, Silverstone SE (1991) Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. Appl Environ Microbiol 57:535–538

Brown ME, Burlingham SK (1968) Production of plant growth substances by *Azotobacter chroococcum*. J Gen Microbiol 53:135–144

Cappucino JC, Sherman N (1992) Nitrogen cycle. In: Microbiology: a laboratory manual, 4th edn. Benjamin/Cumming, New York, pp 311–312

Compant S, Duffy B, Nowak J, Clément C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 71:4951–4959

Conn VM, Franco CM (2004) Effect of microbial inoculants on the indigenous actinobacterial endophyte population in the roots of wheat as determined by terminal restriction fragment length polymorphism. Appl Environ Microbiol 70:6407–6413

Coroler L, Elomari M, Hoste B, Gillis M, Izard D, Leclerc H (1996) *Pseudomonas rhodesiae* sp. nov., a new species isolated from natural mineral waters. Syst Appl Microbiol 19:600–607

Dey R, Pal K, Bhatt D, Chauhan S (2004) Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. Microbiol Res 159:371–394

Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res 17:7843–7853

Ghosh S, Penterman JN, Little RD, Chavez R, Glick BR (2003) Three newly isolated plant growth-promoting bacilli facilitate the seedling growth of canola. Brassica campestris. Plant Physiol Biochem 41: 277–281

Goldstein AH (1994) Involvement of the quinoprotein glucose dehydrogenase in the solubilization of exogenous phosphates by gram-negative bacteria. In: Torriani-Gorni A, Yagil E, Silver S (eds) Phosphate in microorganisms: cellular and molecular biology. ASM Press, Washington, pp 197–203

Grover M, Ali SZ, Sandhya V, Rasul A, Venkateswarlu B (2011) Role of microorganisms in adaptation of agriculture crops to abiotic stresses. World J Microbiol Biotechnol 27:1231–1240

Gulati A, Rahi P, Vyas P (2008) Characterization of phosphate-solubilizing fluorescent pseudomonads from the rhizosphere of seabuckthorn growing in the cold deserts of Himalayas. Curr Microbiol 56:73–79

Gupta HS, Kant L (2012) Wheat improvement in Northern hills of India. Agric Res 1:100–116

Han SO, New P (1998) Variation in nitrogen fixing ability among natural isolates of *Azospirillum*. Microb Ecol 36:193–201

Hantsis-Zacharov E, Halpern M (2007) Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. Appl Environ Microbiol 73:7162–7168

Holland MA, Polacco JC (1994) PPFMs and other covert contaminants: is there more to plant physiology than just plant? Annu Rev Plant Biol 45:197–209

Holland MA, Davis R, Moffitt S, O'Laughlin K, Peach D, Sussan S, Wimbrow L, Tayman B (2000) Using "leaf prints" to investigate a common bacterium. Am Biol Teach 62:128–131

Hu X, Chen J, Guo J (2006) Two phosphate and potassium-solubilizing bacteria isolated from Tianmu mountain, Zhejiang, China. World J Microbiol Biotechnol 22:983–990

Jaccard P (1912) The distribution of the flora in the alpine zone. New Phytol 11:37–50

Jacobson CB, Pasternak J, Glick BR (1994) Partial purification and characterization of 1-aminocyclopropane-1-carboxylate deaminase from the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. Can J Microbiol 40:1019–1025

Jha P, Kumar A (2009) Characterization of novel plant growth promoting endophytic bacterium *Achromobacter xylosoxidans* from wheat plant. Microb Ecol 58:179–188

Kloepper J, Schroth M, Miller T (1980) Effects of rhizosphere colonization by plant growth-promoting rhizobacteria on potato plant development and yield. Phytopathology 70:1078–1082

Kumar M, Yadav AN, Tiwari R, Prasanna R, Saxena AK (2013) Deciphering the diversity of culturable thermotolerant bacteria from Manikaran hot springs. Ann Microbiol 64:741–751

Kumar M, Yadav AN, Tiwari R, Prasanna R, Saxena AK (2014) Evaluating the diversity of culturable thermotolerant bacteria from four hot springs of India. J Biodivers Bioprospect Dev 1:127. doi:10.4172/ijbbd.1000127

Liu Y, Zuo S, Zou Y, Wang J, Song W (2013) Investigation on diversity and population succession dynamics of endophytic bacteria from seeds of maize (*Zea mays* L., Nongda108) at different growth stages. Ann Microbiol 63:71–79

Madhaiyan M, Poonguzhali S (2014) *Methylobacterium pseudosasicola* sp. nov. and *Methylobacterium phyllostachyos* sp. nov., two novel species isolated from bamboo leaf surfaces. Int J Syst Evol Microbiol 64:2376–2384. doi:10.1099/ijs.0.057232-0

Magnani G, Didonet C, Cruz L, Picheth C, Pedrosa F, Souza E (2010) Diversity of endophytic bacteria in Brazilian sugarcane. Genet Mol Res 9:250–258

McInroy JA, Kloepper JW (1995) Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. Can J Microbiol 41: 895–901

Mehta S, Nautiyal CS (2001) An efficient method for qualitative screening of phosphate-solubilizing bacteria. Curr Microbiol 43:51–56

Misaghi I, Donndelinger C (1990) Endophytic bacteria in symptom-free cotton plants. Phytopathology 80:808–811

Mishra PK, Bisht SC, Ruwari P, Selvakumar G, Joshi GK, Bisht JK, Bhatt JC, Gupta HS (2011) Alleviation of cold stress in inoculated wheat (*Triticum aestivum* L.) seedlings with psychrotolerant Pseudomonads from NW Himalayas. Arch Microbiol 193:497–513

Monib M, Zahra MK, Abdel Al, Heggo A (1984) Role of silicate bacteria in releasing K and Si from biotite and orthoclase. Soil biology and conservation of the biosphere. In: Szegi, J. (ed) Akademiai Kiado, Budapest, pp 733–743

Murphy J, Riley J (1962) A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta 27: 31–36

Nejad P, Johnson PA (2000) Endophytic bacteria induce growth promotion and wilt disease suppression in oilseed rape and tomato. Biol Control 18:208–215

Pandey S, Singh S, Yadav AN, Nain L, Saxena AK (2013) Phylogenetic diversity and characterization of novel and efficient cellulase producing bacterial isolates from various extreme environments. Biosci Biotechnol Biochem 77:1474–1480

Patten CL, Glick BR (2002) Role of Pseudomonas putida indoleacetic acid in development of the host plant root system. Appl Environ Microbiol 68:3795–3801

Rico A, Ortiz-Barredo A, Ritter E, Murillo J (2004) Genetic characterization of *Erwinia amylovora* strains by amplified fragment length polymorphism. J Appl Microbiol 96:302–310

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Saravanan VS, Osborne J, Madhaiyan M, Mathew L, Chung J, Ahn K, Sa T (2007) Zinc metal solubilization by Gluconacetobacter diazotrophicus and induction of pleomorphic cells. J Microbiol Biotechnol 17:1477–1482

Schwyn B, Neilands J (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

Selvakumar G, Joshi P, Suyal P, Mishra PK, Joshi GK, Bisht JK, Bhatt JC, Gupta HS (2011) *Pseudomonas lurida* M2RH3 (MTCC 9245), a psychrotolerant bacterium from the Uttarakhand Himalayas, solubilizes phosphate and promotes wheat seedling growth. World J Microbiol Biotechnol 27:1129–1135

Sheng XF (2005) Growth promotion and increased potassium uptake of cotton andrape by a potassium releasing strain of*Bacillus edaphicus*. Soil Biol Biochem 37:1918–1922

Sijam K, Dikin A (2005) Biochemical and physiological characterization of *Burkholderia cepacia* as biological control agent. Int J Agric Biol 7:385–388

Sturz A, Christie B, Matheson B, Arsenault W, Buchanan N (1999) Endophytic bacterial communities in the periderm of potato tubers and their potential to improve resistance to soil-borne plant pathogens. Plant Pathol 48:360–369

Sun L, Lu Z, Bie X, Lu F, Yang S (2006) Isolation and characterization of a co-producer of fengycins and surfactins, endophytic *Bacillus amyloliquefaciens* ES-2, from *Scutellaria baicalensis* Georgi. World J Microbiol Biotechnol 22:1259–1266

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Thomas P, Soly TA (2009) Endophytic bacteria associated with growing shoot tips of banana (*Musa* sp.) cv. Grand naine and the affinity of endophytes to the host. Microb Ecol 58:952–964

Tilak KVBR, Ranganayaki N, Pal KK, De R, Saxena AK, Nautiyal CS, Mittal S, Tripathi AK, Johri BN (2005) Diversity of plant growth and soil health supporting bacteria. Curr Sci 89:136–150

Verma P, Yadav AN, Kazy SK, Saxena AK, Suman A (2014) Evaluating the diversity and phylogeny of plant growth promoting bacteria associated with wheat (*Triticum aestivum*) growing in central zone of India. Int J Curr Microbiol Appl Sci 3:432–447

Vyas P, Rahi P, Gulati A (2009) Stress tolerance and genetic variability of phosphate-solubilizing fluorescent *Pseudomonas* from the cold deserts of the trans-Himalayas. Microb Ecol 58:425–434

Walkley A, Black IA (1934) An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci 37:29–38

Yadav AN, Sachan SG, Verma P, Saxena AK (2014a) Prospecting cold deserts of north western Himalayas for microbial diversity and plant growth promoting attributes. J Biosci Bioeng. doi:10.1016/j.jbiosc.2014.11.006

Yadav AN, Sachan SG, Verma P, Tyagi SP, Kaushik R, Saxena AK (2014b) Culturable diversity and functional annotation of psychrotrophic bacteria from cold desert of Leh Ladakh (India). World J Microbiol Biotechnol. doi:10.1007/s11274-014-1768-z

Yadav AN, Verma P, Kumar M, Pal KK, Dey R, Gupta A, Padaria JC, Gujar GT, Kumar S, Suman A, Prasanna R, Saxena AK (2014c) Diversity and phylogenetic profiling of niche-specific Bacilli from extreme environments of India. Ann Microbiol. doi:10.1007/s13213-014-0897-9

Yang J, Kloepper JW, Ryu C-M (2009) Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci 14:1–4

Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kuczmarski D, Higley P, Ishimaru CA, Arunakumari A, Barletta RG, Vidaver AK (2002) Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl Environ Microbiol 68:2198–2208