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Prospecting cold deserts of north western Himalayas for microbial diversity and plant growth promoting attributes

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Microbial communities in different samples collected from cold deserts of north western Himalayas, India, were analyzed using 16S rRNA gene sequencing and phospholipid fatty acids (PLFA) analysis. A total of 232 bacterial isolates were characterized employing 16S rDNA-Amplified Ribosomal DNA Restriction Analysis with the three restriction endonucleases *Alu* I, *Msp* I and *Hae* III, which led to formation of 29–54 groups for the different sites, adding up to169 groups. 16S rRNA gene based phylogenetic analysis, revealed that 82 distinct species of 31 different genera, belonged to four phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. PLFA profiling was performed for concerned samples which gave an estimate of microbial communities without cultivating the microorganisms. PLFA analysis led to characterization of diverse group of microbes in different samples such as gram-negative, gram-positive bacteria, actinomycetes, cyanobacteria, anaerobic bacteria, sulphate reducing bacteria and fungi. The representative strains were screened for their plant growth promoting attributes, which included production of ammonia, HCN, gibberellic acid, IAA and siderophore; solubilization of phosphorus and activity of ACC deaminase. *In vitro* antifungal activity assay was performed against *Rhizoctonia solani* and *Macrophomina phaseolina.* Cold adapted microorganisms may serve as inoculants for crops growing under cold climatic conditions. To our knowledge, this is the first report for the presence of *Arthrobacter nicotianae*, *Brevundimonas terrae*, *Paenibacillus tylopili* and *Pseudomonas cedrina* in cold deserts and exhibit multifunctional PGP attributes at low temperatures.

[**Key words:** Cold desert; Psychrotrophic bacteria; North western Himalayas; Plant growth promotion; Phospholipid fatty acid; Culture-dependent; Culture-independent]

Earth is primarily a cold, marine planet with 90% of the ocean's waters being at 5°C or lower. Much of the oceans, which cover some 70% of the Earth's surface, are at an average temperature of −1°C to +5°C (1). Habitats for cold adapted microorganisms represent a large proportion of the earth's area. Permafrost soils, glaciers, ice sheets and snow cover constitute 20% of the Earth's surface area (2). Alpine and Polar region constitute 25% of the world's land surface area. Prospecting the cold habitats has led to the isolation of a great diversity of microorganisms and has been extensively investigated in the past few years with a focus on culture-dependent techniques (3,4). Many bacteria have been isolated from cold environments such as *Arthrobacter, Pseudomonas, Janthinobacterium, Sphingobacterium, Exiguobacterium, Planococcus, Psychrobacter, Sporosarcina* and *Paenibacillus glacialis* (3,5,6). North western Himalayas are characterized by low temperatures and represent habitat which could be a valuable source for cold adapted microorganisms involved in nutrient cycling and contributing to soil structure and fertility (3,7).

It has been suggested that at least 99% of bacteria observed under a microscope are not cultured by common laboratory techniques. To overcome problems associated with non-culturable microorganisms, various methods have been developed to identify and study these microorganisms. Phospholipid fatty acid (PLFA) profiles offer sensitive reproducible measurements for characterizing the numerically pre-dominant soil microbial communities without cultivating the organisms. The technique provide estimates of both microbial community composition and biomass size (8). This analysis quantifies fatty acids in membrane structures of organisms belonging to different taxonomic groups (Eubacteria, cyanobacteria, fungi, and other eukaryotic organisms) (9). The presence and relative abundance of fatty acids unique to these groups allows the generation of whole community profiles based on a taxonomic, rather than functional parameters.

Plant growth promoting bacteria (PGPB) have been shown to promote plant growth directly, e.g., by fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, potassium and zinc, production of siderophores and plant growth hormones such as cytokinins, auxins and gibberellins. Several bacteria support plant growth indirectly, via production of antagonistic substances or by inducing resistance against plant pathogens (7,10,11). By producing plant growth regulators, these organisms are reported to influence seed germination and seedling growth (7). The genera of PGPB found included, *Arthrobacter, Bacillus, Brevundimonas, Burkholderia, Pseudomonas, Citricoccus, Exiguobacterium, Flavobacterium, Janthinobacterium, Kocuria, Lysinibacillus, Methylobacterium, Microbacterium, Paenibacillus, Providencia* and *Serratia* (7,12,13). Among these genera,

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Pseudomonas and *Exiguobacterium* are best characterized for plant growth promotion (PGP) at low temperatures (7,10). Several studies have demonstrated the benefits of PGPB on the growth and yield of different crops at different climates, soils, and temperatures. Psychrotolerant microorganisms can be valuable in agriculture as inoculants (PGPB) and biocontrol agents in low temperature habitats. The use of psychrophiles as biofertilizers, biocontrol agent and bioremediators would be of great use in Indian agriculture under cold climatic conditions. The present investigation deals with identification of microorganism using culture-dependent method of 16S rRNA gene sequencing and culture-independent methods by lipid biomarker analysis. Following culturable technique, isolation, characterization, phylogenetic analysis and the plant growth promoting potential of cold adapted culturable microbes was performed for samples collected from north western (NW) Himalayas, India.

MATERIALS AND METHODS

Study sites and sample collection Water, soil and sediment samples were collected from different sites of cold deserts of NW Himalayas, which includes Chandratal Lake (32° 28′ 31″N:77° 37′ 02″E), Rohtang Pass (32° 22′ 17″N:77° 14′ 47″E), Dashair Lake (32° 22′ 17″N:77° 14′ 47″E) and Beas River (32° 21′ 59″N:77° 05′ 08″E), details of which are provided in Table 1. Chandratal Lake is situated at a height of about 4250 m (13,900 ft) in Spiti district of Himachal Pradesh and lies between a low ridge and the main Kunzum range. It is a deep blue-water lake surrounded with snow having circumference of 2.5 km. Chandratal Lake (Moon Lake) is the source of river Chandra. Dashair Lake (Sarkund Lake) is a glacial lake of depth 3 km, situated at a height of about 4270 m and lies at 152–183 m above the Rohtang Pass. The Beas River rises 4361 m above sea-level on the southern face of Rohtang Pass in Kullu. A total of thirty-six samples were collected from four different sites of NW Himalayas. Soil samples from Rohtang Pass were collected from different altitudes (3978–4069 m). Prior to collection, 1 cm of the surface soil was removed with a sterile spatula and using another sterile spatula the soil was collected and transferred into sterile polythene bags. Samples from the Lakes comprised of three samples, each of surface water (10–60 cm from the surface), sub-surface water (100–200 cm from surface) and deep sediments (10–50 cm from the bottom) collected in sterilized labelled bottles, transported on ice and stored at −20°C until analysis. The samples collected from the same site were pooled for further analyses.

Physico-chemical properties of samples The temperature was measured on site by using a scientific thermometer. Soil pH and conductivity was measured using a soil-water mixture 1:5 (w/v) with a pH meter and conductivity meter (Thermo Scientific, Beverly, MA, USA), respectively. Total nitrogen (%) was analyzed using Kjeldahl's procedure by N-analyzer UDK-149, VELP Scientifica Srl, Italy (14). Soil samples were analyzed for soil organic carbon according to Walkley and Black's rapid titration method (15). Soil microbial activity was determined by dehydrogenase activity, which is a measurement of soil microbial oxidation. The dehydrogenase and alkaline phosphatases activities were analyzed according to methods described by Casida et al. (16) and Tabatabai and Bremner (17), respectively.

Microbial enumeration and characterization The population of culturable psychrophilic and psychrotolerant bacteria in the water, sediments and soil samples were enumerated through enrichment method using the standard serial dilution plating technique. Eleven different nutrient media were used to isolate the maximum possible culturable morphotypes (Supplementary Table S1). Full strength, 10, 50 and 100 times diluted medium was used to isolates oligotrophic microorganisms (18). Water samples were filtered through 0.22 μm membranes filters and isolation was done using imprinting method. Low temperature adapted endospore formers were isolated by modified heat enrichment technique as described earlier (19). The plates were incubated at 4°C and the population was counted after 15–60 days (20). Colonies that appeared were purified by repeated streaking to obtain isolated colonies using respective medium plates. The pure cultures were maintained at 4°C as slant and as glycerol stock (20%) at −80°C for further use. All the isolates were screened in triplicates for tolerance to temperature, salt and pH as method described earlier (19).

PCR amplification of 16S rRNA gene and amplified rDNA restriction analysis Genomic DNA was extracted by the method as described earlier (21) with minor modification in the protocol. The amount of DNA extracted was electrophoresed on 0.8% agarose gel. Amplification of 16S rRNA gene was done by using the universal primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) (22). The amplification was carried out in a 100 μL volume and amplification conditions were used as described earlier (23). The PCR amplified 16S rDNA was purified with a Quiaquick purification kit (Qiagen). Aliquots of purified 16S rDNA PCR products were digested separately with three restriction endonucleases *Alu* I, *Hae* III and *Msp* I in 25 μL reaction volumes, using the manufacturer's recommended buffer and temperature. The clustering analysis was undertaken using the software, NTSYS-2.02e package (Numerical Taxonomy System program package, Exeter Software, USA). Similarity among the isolates was calculated by Jaccard's coefficient and dendrogram was constructed using the UPGMA method.

16S rRNA gene sequencing and phylogenetic analysis PCR products of partial 16S rRNA gene of one representative strain from each amplified rDNA restriction analysis (ARDRA) were sequenced with fluorescent terminators (Big Dye, Applied Biosystems) and run in 3130xl Applied Biosystems ABI prism automated DNA sequencer at SCI Genome Chennai, India. The 16S rRNA sequences were aligned using the multiple alignment program Clustal W and the consensus sequence generated was checked for chimeric artefacts with the Check Chimera program available in the Ribosomal Database Project. The sequences were compared with the NCBI GenBank database, using the BLASTn program available in the National Centre for Biotechnology Information (NCBI). Bacterial isolates were identified based on the percentage of sequence similarity (≥97%) with that of a prototype strain sequence available in the GenBank. The phylogenetic tree was constructed using the aligned datasets with the Neighbor Joining method implemented in the program MEGA 4.0.2 (24).

Accession numbers The partial 16S rRNA gene sequences of 169 strains were submitted to NCBI GenBank and accession numbers assigned were JX428963-66, JX428968, JX429005-17, JX429019-25, JX429027-28, JX429031-32, JX429034-48, JX429052-57, JX429059-66, JX429068, JX429071, JX460851, KF054982-86, KF672712-27, KM878131-216 and KJ475009. All 169 isolates were deposited at culture collection facility of National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau, Uttar Pradesh, India.

Phospholipid fatty acid analysis The microbial community composition was determined on the basis of the phospholipid fatty acid (PLFA) pattern (25). PLFA analysis was carried out by KAD Bioresources Pvt Ltd., Ahmedabad. The different fatty acids were used for the determination of the notional groups of gram-positive bacteria, gram-negative bacteria, actinobacteria, fungi, protozoa, yeasts, anaerobic bacteria, *Pseudomonas, Methylobacterium* and *Arthrobacter* (9). The PLFA nomenclature follows the pattern of A:BωC as described by Oravecz et al. (26). The ratio of total monounsaturated/total saturated fatty acid (MUFA/STFA) was used to identify substrate availability for microbes in sediments (27).

Statistical analysis In order to compare the bacterial diversity within the four sites, the 16S rRNA gene sequences and fatty acid biomarkers composition data was used to analyze diversity index. The software Shannon–Wiener Diversity Index/Shannon entropy calculator was used to calculate the Shannon index (H), Evenness (J) and the Simpson's index (D).

Screening for plant growth promoting attributes Representative strains from each cluster were screened for their PGP attributes. Phosphate solubilizing activity of the isolates were studied on Pikovskaya agar (28). Siderophore production was examined on chrome azurol-S agar medium as described by Schwyn and Neilands (29). IAA and gibberellic acid production was tested according to the method of Bric et al. (30) and Brown and Burlingham (31), respectively. Production of ammonia was tested as per procedure of Cappucino and Sherman (32). HCN production was checked by the method described by Bakker and Schippers (33). The bacterial strains were screened for their ability to utilize 1-aminocyclopropane-1-carboxylate (ACC) as sole nitrogen source, a trait which is a consequence of the activity of the enzyme ACC deaminase (34). All assays were done in triplicate at 4°C, 15°C and 30°C.

Quantitative estimation of phosphate solubilization was performed by the ascorbic acid method as described by Murphy and Riley (35). Indole acetic acid production was estimated by inoculating a bacterial suspension of 1 mL aliquots (3×10^9 cfu/mL) in 50 mL Luria Bertani (LB) broth containing L tryptophan (100 μg/mL) and incubated at 4°C, 15°C, and 30°C for 14 days. The IAA concentration in the culture supernatant was estimated by the procedure of Gordon and Weber (36).

TABLE 1. Geographical details and physico-chemical properties of samples and sampling sites.

Sampling location	No. of sample	Temperature (°C)	pH	Conductivity (mS cm^{-1})	Total N (%)	Organic C (%)	Dehydrogenase (μg g^{-1}dry soil h^{-1})	Alkaline phosphatase (μg ml^{-1} h^{-1})
Chandratal Lake	9	−20 − +10	6.8–8.9	0.578 − 1.252	0.9 ± 0.2	3.12 ± 0.1	2.25 ± 0.02	0.95 ± 0.04
Rohtang Pass	12	−10 − +10	6.5–8.2	0.030 − 0.232	1.4 ± 0.1	4.61 ± 0.2	3.86 ± 0.07	1.93 ± 0.02
Dashair Lake	9	−10 − +15	6.9–8.3	0.078 − 0.837	1.0 ± 0.2	3.56 ± 0.3	2.32 ± 0.02	1.88 ± 0.06
Beas River	6	−10 − +10	6.7–8.4	0.086 − 0.352	0.8 ± 0.3	2.45 ± 0.2	1.64 ± 0.11	0.68 ± 0.07