



Evaluating the Diversity of Culturable Thermotolerant Bacteria from Four Hot Springs of India

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Abstract

The culturable bacterial diversity of four hot springs of India was analysed, employing different media and screened for temperature tolerance (40°C - 70°C). Sixty morphotypes from Bakreshwar, 48 from Balrampur, 46 from Chumathang and 52 from Vashist were obtained. A set of 112 isolates, tolerant to 45°C and above, were analysed employing Amplified ribosomal DNA restriction analysis (ARDRA). Sequencing of 16S rRNA gene of the representative isolates revealed that 86%, 93%, 44% and 44% of the isolates respectively from Bakreshwar, Balrampur, Chumathang and Vashist, belonged to Firmicutes. Members of Actinobacteria were present in all the four hot springs, while Proteobacteria were present only in Chumathang and Vashist and Bacteroidetes found only in Bakreshwar. This is the first report of *Aurantimonas* and *Brevundimonas* in hot springs. Biolog analyses of three isolates, growing at or above 60°C revealed unique abilities, in terms of utilization of substrates and resistance patterns.

Keywords: ARDRA; Biolog; Culturable bacteria; 16S rRNA; Hot springs; Thermotolerance

Abbreviations

ARDRA: Amplified ribosomal DNA restriction analysis; rRNA: Ribosomal ribonucleic acid; PCR: Polymerase chain reaction; HGB: Himalayan geothermal belt

Introduction

Among the many extreme environments, thermal springs are of considerable interest to researchers worldwide. Microbial communities present in such habitats constitute valuable sources for various biotechnological products [1,2]. India is the home to several hot springs, which have been less explored in terms of biotic components [3], as there are very limited published literatures available on the microbial diversity and their utility [3,4].

Worldwide, studies on microbial communities in hot springs have mainly concentrated on habitats at low elevations like Yellowstone National Park [5], Kamchatka in Russia, Iceland [6], Indonesia [7] and Tunisia [2]. But, very little is known about the bacterial diversity in thermal springs from highly elevated regions. The Himalayan Geothermal Belts (HGB) contains nearly 150 thermal springs. Chumathang in Jammu and Kashmir and Vashist in Himachal Pradesh [8] representing high altitude are well known hot springs of HGB. Bakreshwar is a well known hot spring representing plain located in West Bengal which shows temperature in the range of 35°C to 66.5°C [9]. Balrampur, located in Chattisgarh is the home to a low altitude hot spring, which is less investigated. In the present investigation, an attempt was made to compare the diversity of culturable bacteria in these four hot springs and identify niche-specific bacterial genera/groups.

In the recent years there has been an increasing emphasis on metagenomic approaches to analyse diversity [2,10,11]. Traditional methods of isolation and culturing have several limitations and can be considered inefficient in a way that they cannot recover symbiotic, slow growing organisms and viable but non-culturable fractions that are believed to make up the bulk of environmental samples [12]. Despite limitations, the advantage of culture based diversity studies outweighs metagenomic approaches in the opportunity it offers to generate a large and valuable germplasm. Also, the main advantage in working with isolated strains is that they can be preserved for further studies and can be explored for biotechnological applications, when the need arises [13,14].

Advances in molecular biology techniques such as sequencing a stable part of genetic code have provided an excellent opportunity for complementing the identification and characterization of bacteria at species and subspecies levels [15]. Candidate genes used for this study included as many as 20 including 5S rRNA, 16S rRNA, 23S rRNA and 16-23S rRNA internal transcribed spacer (ITS) region. Among all these genes, 16S rRNA is considered the best evolutionary chronometer because (i) it is universally present in all bacteria; (ii) its function over time has not changed, suggesting that random sequence changes are a more accurate measure of time (i.e. evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for bioinformatic analyses [16]. Amplified ribosomal DNA restriction analysis (ARDRA) represents a further improvement in the analysis of 16S rRNA genes and involves amplification of 16S rRNA gene followed by digestion of amplified product using selected restriction enzymes and generation of restriction patterns. Restriction patterns can then be compared [17] and analysed using bioinformatic tools. The utility of ARDRA in genotypic characterization of bacteria [18] and screening clone libraries [19] for grouping them into phylogenetic clusters is undisputed. ARDRA has been utilized in the past for identification of bacteria up to species level also [20].

In the present investigation on diversity of thermotolerant bacteria in four hot springs of India (Bakreshwar, Balrampur, Chumathang and Vashist), a culture based method complementing with ARDRA and 16S rRNA gene sequencing was used. Niche specific taxonomic groups of bacteria were defined. A set of selected highly thermo tolerant strains were further characterized using Biolog.

Materials and Methods

Sampling and Isolation of bacteria

Water samples were collected from four hot springs and brought to the laboratory in thermos flasks within 12 hours. These samples were then processed for the isolation of culturable bacteria. The physico-chemical characteristics of the water samples were tested in terms of pH and temperature. Five different media were used for enumeration and isolation, using standard spread plate technique and incubated at 37°C for 48-72 h. The composition of the different media employed were - Nutrient Agar (Peptone 0.5%, Beef extract 0.3%, NaCl 0.5% and Agar 1.8%), Thermus Agar (Peptone 0.5%, Yeast extract 0.2%, Beef extract 0.4%, NaCl 0.5% and Agar 1.8%), R2A medium (Proteose Peptone 0.05%, Casamino acid 0.05%, Yeast extract 0.05%, Dextrose 0.05%, Soluble starch 0.05%, Dipotassium hydrogen phosphate 0.03%, Sodium Pyruvate 0.03%, Magnesium sulphate heptahydrate 0.005%), King's B medium (Protease Peptone 2%, Dipotassium hydrogen phosphate 0.15%, Magnesium sulphate 0.15% and Agar 1.8%) and Thermus Peptone Meat extract Yeast extract medium (TPMY : Peptone 0.35%, Meat extract 0.5% Yeast extract 0.2% , NaCl 0.15% and Agar 1.8%). The total viable count was recorded in the different media.

Screening for temperature tolerance

All the isolates from each of the four springs were screened for temperature tolerance by spotting the cultures on specific media at different temperatures viz 40, 45, 50, 55 and 60°C for 72 h. Isolates growing at 60°C on solid media were tested further at temperatures beyond 60°C in liquid media by incubating them in water bath-shaker.

Molecular techniques and Bioinformatics analysis

Methods of DNA extraction, PCR amplification of 16S rRNA gene, ARDRA, 16S rRNA gene sequencing and phylogenetic analysis were as followed in our earlier studies [21].

Biolog analyses of selected isolates

A set of isolates capable of growing upto a temperature of 60° C were characterized using the GEN III MicroPlate™, to generate a profile based on 94 biochemical tests (70 carbon sources and 22 chemical sensitivity tests). Biolog's microbial identification system software was used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate.

Results and Discussion

Hot springs are manifestations of geological activity and represent extreme environments which have been mainly explored in terms of their physico-chemical characteristics. Among the different extreme environments, thermal springs are analogous to primitive earth and hydrothermal processes on Mars. Hence, the microbial communities thriving in geothermal hot springs have been the subject of extensive

research [22]. Ever since Thomas Brock discovered the presence of *Thermus aquaticus* in the thermal vents of Yellowstone National Park, a number of research groups started exploring similar environments all around the world [2,5-7,23]. Despite intensive studies on geothermal springs, scanty information is available on diversity of bacteria in moderate altitudes or high altitude regions.

Indian subcontinent is a host to innumerable number of geothermal hot springs, especially the Himalayan Geothermal Belt which contains close to 150 thermal springs. Chumathang which is at an elevation of 4023 m above mean sea level and Vashist, which is at an elevation of 1982 m come under HGB. Bakreshwar hot springs present in eastern India, is at an elevation of 84 m above mean sea level and Balrampur hot springs present in central India, is at an elevation of 623 m above mean sea level. Our investigation focussed on the diversity of culturable thermotolerant bacteria from hot springs present in both the high altitude regions and plains. Chumathang, Vashist and Balrampur hot springs represent high altitude regions in the descending order of altitude and Bakreshwar represents the geothermal springs of plains (Figure 1). Among the different samples, the temperature was highest in the Balrampur hot springs and lowest in Vashist hot springs.

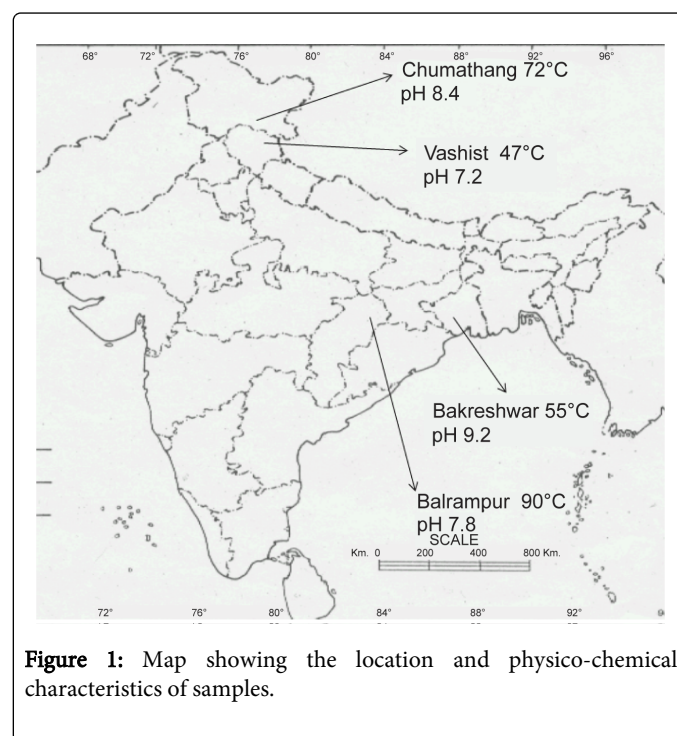


Figure 1: Map showing the location and physico-chemical characteristics of samples.

Enumeration and isolation of culturable bacteria

Five different types of media were employed to capture the maximum possible diversity. These included a nutrient rich medium (Nutrient Agar) suitable for most nutritional types, a medium suitable for oligotrophs (R2A), specific media designed for thermophiles (Thermus Agar and Thermus Peptone Meat extract Yeast extract medium) and a medium mostly used for *Pseudomonas* and related genera (King's B medium). Enumeration of bacterial population revealed that aerobic heterotrophic bacteria in Bakreshwar thermal springs ranged from 119 to 183 × 10⁴ CFU/mL and a similar trend was

recorded for Vashist thermal springs, which was in the range of 158 to 229 × 10⁴ CFU/mL. Among the different media used, R2A yielded highest population of 229 × 10⁴ CFU/mL from Vashist samples and for Bakreshwar samples, while TPMY yielded the highest population of 183 × 10⁴ CFU/mL. In the samples from Balrampur and Chumathang, the population of aerobic heterotrophs were in the range from 89 to 193 × 10³ CFU/mL and 69 to 112 × 10⁴ CFU/mL respectively. Thermus agar yielded highest population from both Balrampur and Chumathang samples. A total of 60, 48, 46 and 52 morphotypes were selected from Bakreshwar, Balrampur, Chumathang and Vashist hot spring samples respectively, for further studies.

Screening for temperature tolerance

Screening for temperature tolerance revealed a total of 27, 38, 18, and 29 isolates from Bakreshwar, Balrampur, Chumathang and Vashist respectively were able to grow at 45°C. Seven isolates were able to grow at 50°C, while five were able to grow at 55°C and three were able to grow at 60°C. Only one isolate was able to grow at 70°C. Interestingly, only the isolates from Bakreshwar were able to grow at temperatures beyond 45°C, although Bakreshwar hot springs did not exhibit the highest temperature among the four hot springs. The microbial community obtained from these hot springs includes mesophiles also, which reveals the possibility of beneficial partnerships of such high temperature adapted mesophiles with more thermotolerant non culturable bacteria, as has been recorded in several extreme habitats [5]. This is in consonance with reports regarding the absence of a monotonic relation between temperature stress and microbial community, and illustrating that thermophiles can be members of mesophilic environments also and vice versa [5,24]. Metagenomic analyses can help further throw light on our observations. Isolates which were able to grow at 45°C and above were selected for molecular characterization.

Amplified rDNA restriction analysis

PCR amplification of 16S rRNA gene yielded a single amplicon of 1.5 Kbp from all the isolates. The amplicons were digested with three restriction enzymes. Different patterns comprising of 3 to 6 fragments ranging in size from 100 to 800 bp, were characterized. RFLP analysis revealed that restriction digestion with *Alu I* was more discriminative, as compared to *Msp I* and *Hae III*. A combined dendrogram (based on patterns generated using three enzymes individually) was constructed to determine the percent similarity among the isolates from each of the three thermal springs (data not shown). At 90% similarity level, thermotolerant isolates from Bakreshwar, Balrampur, Chumathang and Vashist thermal springs grouped into 14, 14, 11 and 9 clusters respectively. ARDRA has been effectively utilized in the past for both culture dependent and culture independent diversity studies as a tool to group the isolates and clones into phylotypes [17-19].

16S rRNA gene sequencing and phylogenetic analysis

Representative isolates from each phylogenetic cluster were sequenced and the data were analysed by BLAST and the nearest match from the GenBank database is illustrated (Table 1). Among the 14 isolates sequenced from Bakreshwar hot springs, 10 belonged to the genera *Bacillus*. Sequence results from Chumathang hot springs revealed that 4 out of 11 isolates were *Bacillus*. Two isolates of the 9

representative isolates of Vashist also belonged to *Bacillus*. In Balrampur samples, out of 14 representative isolates, 12 belonged to the genus *Bacillus*. A taxonomic grouping of the isolates revealed that Firmicutes was the dominant division in both Bakreshwar and Balrampur, but Chumathang and Vashist hot water springs exhibited a greater diversity with both containing members from Firmicutes, Proteobacteria and Actinobacteria. Actinobacterial members were present in all the four hot springs studied and Chumathang hot springs recorded highest number i.e. 18% of the sequenced isolates. Members of Proteobacteria were present only in Chumathang and Vashist hot springs and members of Bacteroidetes were found only in Bakreshwar hot springs (Figure 2).

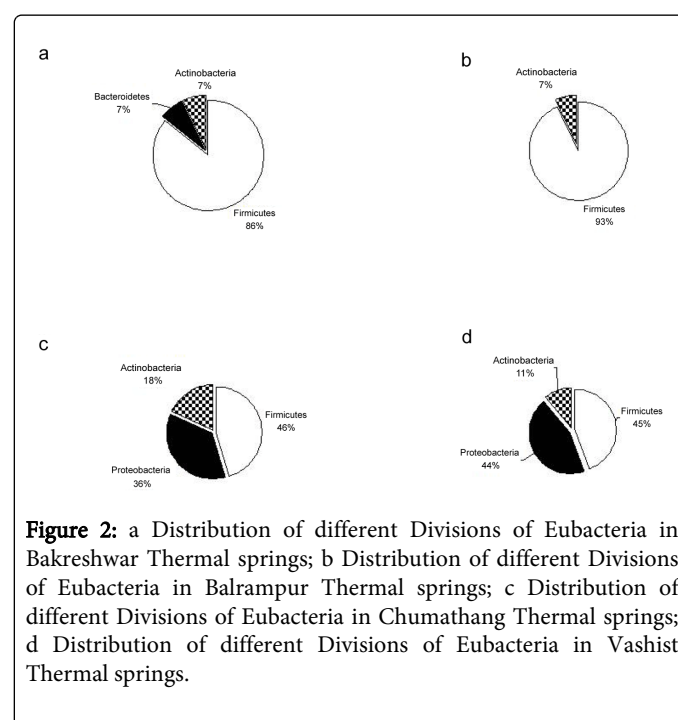


Figure 2: a Distribution of different Divisions of Eubacteria in Bakreshwar Thermal springs; b Distribution of different Divisions of Eubacteria in Balrampur Thermal springs; c Distribution of different Divisions of Eubacteria in Chumathang Thermal springs; d Distribution of different Divisions of Eubacteria in Vashist Thermal springs.

Phylogenetic trees were constructed from the sequences of the representative isolates from each of the four thermal springs along with closest sequences in the NCBI Genebank (Figure 3-6). The use of 16S rRNA gene sequencing in combination with ARDRA for grouping into phylogenetic clusters and identification of bacteria is well documented [18-20]. Although the number of phylogenetic clusters is less in Vashist, the average bacterial population was highest in Vashist and the lowest in Balrampur.

This may be related to the variation in the temperature of the springs, which was least for Vashist and highest for Balrampur. Temperature showed a significant negative correlation with CFU ($r=-0.9950$). It is interesting to note that the pH of Vashist hot spring is also in the neutral range, suggesting the role of temperature and pH in bacterial abundance in geothermal waters, which is in accordance with earlier investigations [25].

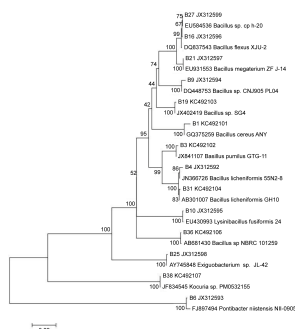


Figure 3: Unrooted phylogenetic tree based on comparison of 16S rDNA sequences of 14 isolates of Bakreshwar along with their closest phylogenetic relatives. Phylogenetic tree was constructed based on aligned datasets using Neighbour joining (NJ) method using the program MEGA 4.0.2. Numbers on the tree indicates percentage of bootstrap sampling derived from 1000 random samples.

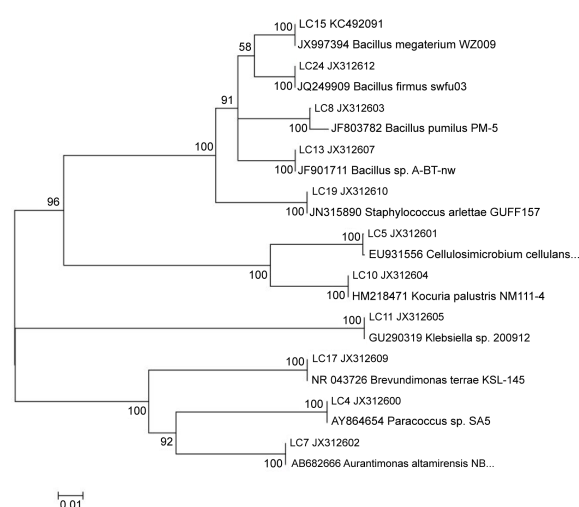


Figure 5: Unrooted phylogenetic tree based on comparison of 16S rDNA sequences of 11 isolates of Chumathang along with their closest phylogenetic relatives. Phylogenetic tree was constructed based on aligned datasets using Neighbour joining (NJ) method using the program MEGA 4.0.2. Numbers on the tree indicates percentage of bootstrap sampling derived from 1000 random samples.



Figure 4: Unrooted phylogenetic tree based on comparison of 16S rDNA sequences of 14 isolates of Balrampur along with their closest phylogenetic relatives. Phylogenetic tree was constructed based on aligned datasets using Neighbour joining (NJ) method using the program MEGA 4.0.2. Numbers on the tree indicates percentage of bootstrap sampling derived from 1000 random samples.

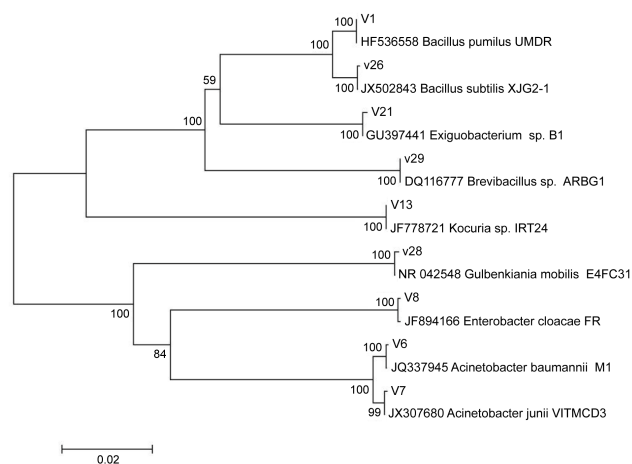


Figure 6: Unrooted phylogenetic tree based on comparison of 16S rDNA sequences of 9 isolates of Vashist along with their closest phylogenetic relatives. Phylogenetic tree was constructed based on aligned datasets using Neighbour joining (NJ) method using the program MEGA 4.0.2. Numbers on the tree indicates percentage of bootstrap sampling derived from 1000 random samples.

RFLP Pattern	Representative isolates	Temperature tolerance	GeneBank accession number	Nearest phylogenetic neighbour	16 S similarity (%)	Division
Bakreshwar						
1	B1	50°C	KC492101	<i>Bacillus cereus</i> (GQ375259)	99	Firmicutes
2	B3	60°C	KC492102	<i>Bacillus pumilus</i> (JX841107)	99	Firmicutes
3	B4	45°C	JX312592	<i>Bacillus licheniformis</i> (JN366726)	99	Firmicutes
4	B6	45°C	JX312593	<i>Pontibacter niistensis</i> (FJ897494)	98	Bacteroidetes
5	B9	45°C	JX312594	<i>Bacillus sp.</i> (DQ448753)	99	Firmicutes
6	B10	45°C	JX312595	<i>Lysinibacillus fusiformis</i> (EU430993)	99	Firmicutes
7	B16	45°C	JX312596	<i>Bacillus flexus</i> (DQ837543)	99	Firmicutes
8	B19	45°C	KC492103	<i>Bacillus sp.</i> (JX402419)	99	Firmicutes
9	B21	50°C	JX312597	<i>Bacillus megaterium</i> (EU931553)	99	Firmicutes
10	B25	55°C	JX312598	<i>Exiguobacterium sp.</i> (AY745848)	99	Firmicutes
11	B27	55°C	JX312599	<i>Bacillus sp.</i> (EU584536)	99	Firmicutes
12	B31	60°C	KC492104	<i>Bacillus licheniformis</i> (AB301007)	99	Firmicutes
13	B36	45°C	KC492106	<i>Bacillus sp.</i> (AB681430)	99	Firmicutes
14	B38	70°C	KC492107	<i>Kocuria sp.</i> (JF834545)	100	Actinobacteria
Balrampur						
1	BC1	JX312575	45°C	<i>Bacillus cereus</i> (JQ739719)	100	Firmicutes
2	BC2	JX312576	45°C	<i>Brevibacillus sp.</i> (AJ313027)	99	Firmicutes
3	BC5	JX312578	45°C	<i>Bacillus pumilus</i> (JQ435673)	99	Firmicutes
4	BC6	JX312579	45°C	<i>Bacillus aryabhatai</i> (HQ242769)	100	Firmicutes
5	BC7	JX312580	45°C	<i>Bacillus firmus</i> (JQ249909)	100	Firmicutes
6	BC9	JX312581	45°C	<i>Bacillus megaterium</i> (EU931553)	100	Firmicutes
7	BC10	JX312582	45°C	<i>Bacillus sp.</i> (HM567109)	100	Firmicutes
8	BC11	JX312583	45°C	<i>Bacillus flexus</i> (HQ875778)	99	Firmicutes
9	BC12	JX312584	45°C	<i>Planococcus sp.</i> (EF471920)	100	Firmicutes
10	BC16	JX312587	45°C	<i>Bacillus licheniformis</i> (GU377281)	99	Firmicutes
11	BC17	JX312588	45°C	<i>Lysinibacillus sp.</i> (FN397524)	100	Firmicutes
RFLP Pattern	Representative isolates	Temperature tolerance	GeneBank accession number	Nearest phylogenetic neighbour	16 S similarity (%)	Division
12	BC21	JX312589	45°C	<i>Staphylococcus haemolyticus</i> (JQ624771)	99	Firmicutes

13	BC27	JX312590	45°C	<i>Rhodococcus sp.</i> (DQ285075)	99	Actinobacteria
14	BC31	JX312591	45°C	<i>Exiguobacterium acetylicum</i> (HM047519)	100	Firmicutes
Chumathang						
1	LC4	45°C	JX312600	<i>Paracoccus sp.</i> (AY864654)	99	Proteobacteria
2	LC5	45°C	JX312601	<i>Cellulosimicrobium cellulans</i> (EU931556)	99	Actinobacteria
3	LC7	45°C	JX312602	<i>Aurantimonas altamirensis</i> (AB682666)	99	Proteobacteria
4	LC8	45°C	JX312603	<i>Bacillus pumilus</i> (JF803782)	99	Firmicutes
5	LC10	45°C	JX312604	<i>Kocuria palustris</i> (HM218471)	100	Actinobacteria
6	LC11	45°C	JX312605	<i>Klebsiella sp.</i> (GU290319)	100	Proteobacteria
7	LC13	45°C	JX312607	<i>Bacillus sp.</i> (JF901711)	100	Firmicutes
8	LC15	45°C	KC492091	<i>Bacillus megaterium</i> (JX997394)	99	Firmicutes
9	LC17	45°C	JX312609	<i>Brevundimonas terrae</i> (NR_043726)	100	Proteobacteria
10	LC19	45°C	JX312610	<i>Staphylococcus arlettae</i> (JN315890)	100	Firmicutes
11	LC24	45°C	JX312612	<i>Bacillus firmus</i> (JQ249909)	100	Firmicutes
Vashist						
1	V1	45°C	KC492092	<i>Bacillus pumilus</i> (HF536558)	100	Firmicutes
2	V6	45°C	KC492093	<i>Acinetobacter baumannii</i> (JQ337945)	99	Proteobacteria
3	V7	45°C	KC492094	<i>Acinetobacter junii</i> (JX307680)	100	Proteobacteria
4	V8	45°C	KC492095	<i>Enterobacter cloacae</i> (JF894166)	100	Proteobacteria
5	V13	45°C	KC492096	<i>Kocuria sp.</i> (JF778721)	99	Actinobacteria
6	V21	45°C	KC492097	<i>Exiguobacterium sp.</i> (GU397441)	99	Firmicutes
7	V26	45°C	KC492098	<i>Bacillus subtilis</i> (JX502843)	99	Firmicutes
8	V28	45°C	KC492099	<i>Gulbenkiania mobilis</i> (NR_042548)	99	Proteobacteria
9	V29	45°C	KC492100	<i>Brevibacillus sp.</i> (DQ116777)	100	Firmicutes

Table 1: RFLP patterns and sequenced representative isolates of thermal springs.

In the present study, 16S rRNA gene sequencing of representative isolates from each phylotype, followed by BLAST search revealed that a majority of sequences from both Balrampur and Bakreshwar (71.4% and 85.7% respectively) showed closeness to *Bacillus*, whereas only 36.4% and 22.2% of the sequenced isolates from Chumathang and Vashist respectively showed closeness to *Bacillus*. Apart from *Bacillus*, representatives from *Bacillus* derived genera were present in both Bakreshwar (*Brevibacillus* and *Lysinibacillus*) and Vashist (*Brevibacillus*) hot springs. The members of the genus *Bacillus* are spore formers and also produce a number of biocidal metabolites/

enzymes which makes them a common inhabitant of diverse extreme habitats [26]. Sufficient evidence exists for reclassification of thermophilic bacteria in the genus *Bacillus* into *Brevibacillus*, *Aneurinibacillus*, *Amphibacillus*, *Virgibacillus*, *Alicyclobacillus*, *Paenibacillus*, *Halobacillus* and *Geobacillus* based on the 16S rRNA gene sequence [15].

A majority of sequenced isolates from all the four hot springs belonged to Firmicutes division. It was found that 86%, 93%, 44% and 44% of the sequenced isolates respectively from Bakreshwar, Balrampur, Chumathang and Vashist belonged to Firmicutes (Figure

2). The bacterial communities in many hot springs are dominated by Firmicutes [10,11,22]. Another commonality in these four hot springs is the presence of isolates belonging to Actinobacteria. Gram positive prokaryotes are known to be comparatively stress resistant and long range migrants, especially the Firmicutes and Actinobacteria [27].

It is interesting to note that bacteria belonging to the division Proteobacteria were isolated from the hot springs in Himalayan Geothermal Belt (Chumathang and Vashist) alone. Bacterial communities in the hot springs of high elevated regions of Tibet are known to be predominated by *Proteobacteria*, alongside Firmicutes [10] which supports our observation. Bacteroidetes is present only in the Bakreshwar which is a representative of plain. The genus in this division is *Pontibacter* which is normally a soil bacterium [28] and has not been isolated from hot springs. The presence of *Pontibacter* suggests a migration of bacterial communities from the sediments to the spring water aided by the flow of springs through the sediments. It is interesting to note that the three hot springs belonging to high altitude region exhibit high level of diversity of bacteria. Chumathang exhibited the highest number of different genera, while Bakreshwar had the least number. This observation supplemented with the presence of *Proteobacteria* in the hot springs of Himalayan Geothermal Belt, reemphasizes the effect of geographic location on the type of bacterial communities in any particular environment [29].

The isolates from Chumathang hot springs showed closeness to many bacteria like *Bacillus* and its derived genera and genera like *Paracoccus*, *Kocuria*, *Klebsiella* and *Staphylococcus*. These genera were also recorded in our earlier study on diversity of thermotolerant bacteria in Manikaran hot springs [21] which also falls under HGB. Besides these, representatives from *Cellulosimicrobium*, *Aurantimonas* and *Brevundimonas* were niche specific to Chumathang hot springs. *Cellulosimicrobium* is very closely related to *Cellulomonas*. Schuman et al. [30] suggested that *Cellulomonas* should be reclassified as *Cellulosimicrobium*. *Cellulomonas* has previously been reported as a culturable thermoresistant aquatic bacterium from the oligotrophic pools in Mexico [27]. To our knowledge, this is the first report on *Aurantimonas* and *Brevundimonas* in a hot spring.

Among the different genera obtained from Vashist hot springs *Exiguobacterium*, *Kocuria* and *Brevibacillus* were reported in our

earlier study on diversity of thermotolerant bacteria from Manikaran [21] and also in the studies on the diversity of culturable thermoresistant aquatic bacteria in Mexico [27]. Representatives from *Acinetobacter*, *Enterobacter* and *Gulbenkiania*, which are pathogens and associated with human intestine, may have been transmitted as contaminants from people taking shower in the spring, as this spring is known for its medicinal properties. The genera *Exiguobacterium*, *Staphylococcus* and *Brevibacillus* were also present in Balrampur hot springs. There are also representations from *Planococcus* and *Rhodococcus*, both being niche specific to Balrampur, but have also been reported in our early study on Manikaran hot springs [21]. Bakreshwar, which showed least diversity with reference to number of genera obtained, has representatives from *Exiguobacterium*, *Kocuria*, *Lysinibacillus* and *Pontibacter*, among which *Pontibacter* was niche specific to Bakreshwar.

Biochemical tests and substrate utilization profiles for selected isolates using Biolog

The data on substrate utilization patterns and sensitivity to chemicals for the 3 temperature tolerant isolates from Bakreshwar were tabulated. Significant variations were observed among the isolates in terms of utilization of sugars, sugar derivatives, metabolic intermediates and amino acids and peptides. Among the three isolates tested for a total of 20 sugars, isolate B31 was able to utilize all, except one sugar. α -D-Glucose, sucrose and pectin were utilized by all the three isolates. Generally known as refractile substrates and not commonly utilized by bacteria, compounds such as D-turanose, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, formic acid and D-serine were not utilized by any of the three isolates. Isolate B31 was able to utilize maximum number of sugar derivatives [15], followed by B3 which was able to utilize 9 of the 19 sugar derivatives tested (Table 2). Out of 19 metabolic intermediates tested, 15 were found utilized by B38 and B31 utilized 13 sugar derivatives (Table 2). The results on amino acids and peptides utilization revealed that the isolate B31 was able to utilize a maximum of 11 amino acids followed by B3, which utilized 7 amino acids (Table 2).

Isolates	Substrates Utilized				Resistance to antimicrobials and growth conditions	
	Saccharides (20)*	Saccharide derivatives (19)	Metabolic intermediates (19)	Amino acids and peptides (12)	Antimicrobial compounds (17)	Growth conditions (5)
B3	9	9	8	7	5	5
B31	19	15	13	11	4	5
B38	9	8	15	2	9	4

Table 2: Profiling of the promising isolates using BIOLOG.

Among the three isolates tested for 17 antimicrobial compounds, the isolate B38 was found to be resistant to nine antimicrobial compounds. All the three isolates exhibited resistance to aztreonam and potassium tellurite. The results also revealed that the isolates were sensitive to five antimicrobial compounds. All the three isolates were able to show growth at pH up to 6 and salt concentration up to 8%.

B38 is the lone isolate which was not able to show growth at pH 5. Biolog based phenotyping of the three highly thermotolerant isolates generated useful unique fingerprints of the isolates (Table 3), besides providing valuable information on the utilization of routine and rare substrates and sensitivity/resistance to antimicrobial compounds.

Among the three isolates, two belonged to the genus *Bacillus* and one belonged to the genus *Turicella*.

Isolates	Substrates	Antimicrobial Compounds	
		Sensitive to	Resistant to
B3	D-Arabitol,	Lithium chloride	Guanidine HCl and Sodium Butyrate
B31	L-Fucose, L-Rhamnose, D-Melibiose, Stachyose, N-Acetyl-β-D-Mannosamine, N-Acetyl-D-Galactosamine, N-Acetyl Neuraminic acid, L-Histidine, L-Pyroglutamic acid, Inosine and Glycyl-L-Proline	-	-
B38	β-Hydroxy - Butyric acid	Sodim lactate	Minocycline, Fusidic acid and Sodium Bromate

Table 3: Unique traits of promising isolates in terms of substrate utilization and response to antimicrobial compounds.

Biolog based identification of temperature tolerant isolates matched with 16S rDNA sequence based identification up to genera level only for two isolates. One isolate for which there was no match was B38, which according to Biolog analysis identifies it as *Turicella otitidis*, but according to 16S rRNA gene sequence, it was placed in the genus *Kocuria*. Morphotyping of isolate B38 revealed its ability to utilize β-hydroxy butyric acid and unique resistance profile against minocycline, fusidic acid and sodium bromate. Earlier studies using have shown its utility in differentiation of *Bacillus* species [31]. However Biolog analyses have certain limitations, as it is unable to distinguish closely related organisms [32]. Also the database of *Actinobacteria* in Biolog is weak hence a clear cut identification of Isolate B38 was difficult; however this strain can have tremendous biotechnological potential.

The identification of isolate B3 using both Biolog analysis and 16S rRNA gene identity matched and placed it as *Bacillus pumilus*. B3 was able to utilize only 35.71% of the substrates tested. Strains of *Bacillus pumilus* generally show high resistance to environmental stresses, including UV light exposure, desiccation, and the presence of oxidizers such as hydrogen peroxide and produce compounds antagonist to fungal and bacterial pathogens [17]. In the present investigation, this strain was able to show growth on 45% of the substrates and chemical agents tested, besides being uniquely able to use D-Arabitol, and uniquely exhibiting resistance to guanidine and sodium butyrate (Table 3).

Isolate B31, identified as *Bacillus licheniformis* based on Biolog, was found to show closest sequence based identity with *Bacillus subtilis*. This isolate was able to uniquely utilize 11 substrates. Rey et al. [33] determined the complete genome sequence of *B. licheniformis* ATCC 14580 and compared the information generated with other species of *Bacillus*, notably with *B. subtilis* 168. Although there exists unmistakable organizational similarities (84.6% identical at the nucleotide level) between the *B. licheniformis* and *B. subtilis* genomes, notable differences in the numbers and locations of prophages, transposable elements and a number of extracellular enzymes and secondary metabolic pathway operons have aided in distinguishing these species. The isolate B31 corresponding to *Bacillus licheniformis* was able to utilize 82.85% of the substrates tested while isolate. In-depth analyses of our isolate may provide more definitive information.

Our study clearly illustrated the significance of evaluating microbial diversity using a combination of traditional morphotyping, followed by molecular approaches and BIOLOG analyses of promising isolates.

Niche specific taxa or genera were also identified. These promising isolates can be of immense significance, as their unique substrate utilization and antibiotic resistance profiles, can make them competitive in the environment.

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