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ORIGINAL ARTICLE

Isolation and characterization of thermophilic bacterial strains from Soldhar (Tapovan) hot spring in Central Himalayan Region, India

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Abstract In the present investigation, 11 morphologically distinct thermophilic bacterial strains have been isolated from the Soldhar hot spring site in the Garhwal region of the Indian Himalaya. The phenotypic and genotypic characters of the isolates were studied using standards methods. All of them developed circular colonies on Tryptone Soy agar plates at 70 °C following 24 h incubation. The isolates grew over a wide range of temperatures (20–100 °C) and pHs (5–10). The cell morphology varied from short to long rods and cocci with a dominance of Gram-positive bacteria. The isolates exhibited varied biochemical properties with regard to the production of various enzymes and capability to ferment sugars. Based on restriction digestion with *Hae*III, six phylogenetic groups were formed. Two isolates were selected for 16S rRNA gene sequence analysis, and exhibited maximum similarity and closest homology with *Geobacillus thermocatenulatus* and *Geobacillus thermoleovorans*, respectively.

Keywords Thermophiles · *Geobacillus* · 16S rRNA · ARDRA · Hot springs · Garhwal Himalaya

Introduction

Microorganisms are found in all possible locations, ranging from those offering ideal conditions for growth and reproduction to extreme environments. This ubiquitous nature of microorganisms is mainly due to small size, easy dispersal, metabolic versatility, ability to tolerate unfavorable conditions and flexibility to utilize a broad range of nutrients. Until recently, most extreme environments were considered too hostile to support any life form, but in the last few decades, it has become evident that they actually provide a natural habitat for certain microorganisms (Islas et al. 2007). The microorganisms that thrive under extreme environmental conditions, from cold deserts to geothermal springs, are known as extremophiles (Seackbach 2000; Staley and Reysenbach 2002). In such extreme environments, the microbial and geochemical interactions are tightly interwoven, providing many of the basic constituents for the primordial synthesis of organic molecules and for the evolution of fundamental metabolic pressure.

The Himalayas have been a perennial source of attraction, curiosity and challenge to the human intellect throughout the ages. Hot water springs are manifestations of geological activity and represent extreme environments that emerge spontaneously in the Himalayas (Kumar et al. 2004). Hot springs have been reported at about 60 locations in the Himalayan region. These hot springs are rich in microbial diversity. Microbial abundance and diversity are influenced by both environmental and biological factors (Liu et al. 2009). Geothermal systems are populated by diverse thermophilic bacteria and archaea (Kaur et al. 2008). Many thermophilic and more than 20 different genera of hyperthermophilic archaea have been isolated from geothermal and hydrothermal environments (Arab et al. 2000). Thermophilic bacilli grow best at temperatures between 45 and 70 °C due to extremozymes, the enzymes geared to work in extremely high temperatures.

A preliminary step in microbiological research is the identification of isolates. For a long time, identification was essentially based on a wide range of biochemical and physiological tests that require the cultivation of bacterial strains, a time-consuming step even when the isolated strains are easy to grow. By contrast, molecular approaches are more sensitive

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and reliable, and they presently dominate modern taxonomic studies as a consequence of technological progress (Vandamme et al. 1996). The sequence comparison of 16S rRNA genes, which are highly conserved throughout prokaryotic organisms, has been most widely used to determine phylogenetic relationships (Staley and Reysenbach 2002; Michaud et al. 2004). An approach using a combination of both methods is needed to obtain objective information about the community composition, and also to evaluate its ecological and physiological function (Vandamme et al. 1996).

The taxonomy and especially the identification of thermophilic bacteria have generated considerable interest over recent decades. The importance of these bacteria has increased, owing to their potential as a source of thermostable enzymes, including xylanases, proteases, amylases, peroxidases, glucose isomerases, lipases and DNA restriction enzymes (Kuisiene et al. 2007; Soliman et al. 2007). Phenotypic and genotypic characterization of thermophilic bacteria has been carried out for many geothermal areas in different parts of the world, including Greece (Sievert et al. 2000), Iceland (Takacs et al. 2001), Italy (Maugeri et al. 2001), Bulgaria (Derekova et al. 2008), India (Sharma et al. 2009), China (Lau et al. 2009) and Turkey (Adiguzel et al. 2009).

The microbial diversity of hot springs in Uttarakhand is presently facing alarming disturbance due to human activities, viz., construction of roads, deposition of soil debris, domestic activities and also natural phenomena like landslides in the Garhwal Himalayas. Moreover, drill holes and wells, constructed for providing hot water to nearby villages, have also reduced the geothermal activity and discharge of hot water. Also, less attention has been paid in the direction of analyzing the microbial diversity of hot spring sites in the Garhwal Himalayan region. Thus, there is an urgent need for isolation, characterization and preservation of the microbial diversity obtained from these hot springs, which will act as a meaningful addition to the database on thermophilic bacterial research. Water and soil samples from the hot spring of Soldhar, located in the Tapovan area of district Chamoli of the Indian Himalayan region, were collected and analyzed for their phenotypic, biochemical and molecular microbial diversity.

Materials and methods

Sampling sites

Water and soil samples were collected from the hot spring of Soldhar (latitude 39° 29′ 25″N, longitude 79° 39′ 29″; altitude 1,900 m amsl), located in the Chamoli district of the Garhwal Himalaya region of Uttarakhand. The approximate area of the site was about 5 m^2. The hot water outlet was present in the middle of the mound at Soldhar and the site was devoid of any vegetation. The samples were collected during October 2012 from the source of the hot water spring, and the pH and temperatures of samples were measured at the sampling site itself. The samples were brought to the laboratory in sterile capped bottles, in thermos flasks. The habitat temperature of samples was maintained during transportation.

Microbial isolation

The samples were serially diluted and plated onto petri plates containing Trypton Soy Agar medium. Inoculated plates were incubated in an incubator for 24 h at 70°C. Colony forming units (CFUs) on each plate were estimated with the help of a colony counter.

Microscopic observations

Representative colonies from agar plates were repeatedly subcultured to obtain pure colonies, which were then subjected to microscopic examination.

Thermophilic bacteria isolated from water and soil samples of Soldhar were characterized for their morphological (colony morphology), microscopic (Gram staining), biochemical (utilization of carbon sources and enzyme activity) and growth (temperature and pH tolerance) characteristics on prescribed media.

Morphological characterization of isolated strains

The colonial morphology of the bacterial isolates was observed on Trypton soy agar medium by direct and stereomicroscopic observations of single colonies. The cell morphology, i.e., size, margin, form, elevation and shape, was studied by the light microscopy of native preparations. Isolates were Gram stained and examined under light microscopy.

Physiological characterization of isolates

The temperature and pH tolerance of isolates was determined by incubating cultures at different temperatures and by modifying the pH of the media. The temperature range for growth was determined by incubating the isolates from 20 to 100 °C with an interval of 10°C. The pH dependence of growth was tested by adjusting the initial pH of the trypton soy agar medium with either 1 M HCl or 1 M NaOH within the range of pH 5.0–10.0, with an interval of 1.0.

Biochemical characterization of isolates

The biochemical characterization was conducted according to the methods of Cappuccino and Sherman (1996). These included Gram staining, gelatin hydrolysis, casein hydrolysis, citrate utilization, methyl red, starch hydrolysis, indole test, H_2S production and carbon utilization tests. Thermophilic

bacterial isolates were also screened for their enzymatic activity (urease and catalase).

DNA extraction and purification

Total bacterial genomic DNA was extracted from each of the test isolates by partial modification of the CTAB chloroform / isoamyl alcohol method (Zhang et al. 2010). Finally, the genomic DNA was eluted with 200 μl of Tris–EDTA buffer (TE) for DNA fingerprinting. DNA was quantified using the Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies, USA), by measuring OD260 and OD280. The quantified DNA samples were diluted in TE buffer to a final concentration of 50 ng/μl for PCR reactions.

16S rRNA gene amplification

The purified DNA samples were used for selective amplification of the 16S rRNA gene using the two universal oligonucleotide primers 27-Fwd 5′AGAGTTTGATCCTGGCTCAG 3′ and 1492-Rev 5′ACGGYTACCTTGTTACGACTT 3′, as described by Zhang et al. (2010), to anneal to conserve positions in the 3′ and 5′ regions of the bacterial 16S rRNA genes. The reaction mixture contained 2.5 μl enzyme buffer, 0.5 μl dNTP, 1 μl 25 pmol forward primer, 1 μl 25 pmol reverse primer, 0.5 μl (5 U) Taq DNA polymerase, and 1 μl DNA sample. PCR amplification was performed in a total volume of 25 μl using thermocycler. The PCR conditions were as follows: initial denaturation for 3 min at 94ºC, and 35 cycles consisting of denaturation at 94ºC for 1 min, primer annealing at 57ºC for 1 min and elongation at 72ºC for 2 min, followed by a final extension at 72ºC for 10 min. The PCR products were visualized by agarose gel electrophoresis on a 0.8 % agarose gel at 80 V for 1 h.

Genetic diversity estimation with amplified ribosomal DNA restriction analysis (ARDRA)

PCR products of the isolates were digested with *Hae*III restriction enzyme (New England Biolabs, Mass., USA). The digestion was carried out in a total volume of 20 μl for 2 h at 40ºC. The mixture contained 15 μl of 1 μg PCR product, 2 μl of 10 X restriction buffer, 1 μl ddH_2O and 2 μl (10 U) of the restriction enzyme. The digested amplicon was visualized by electrophoresis on a 2.5 % low melting agarose gel containing ethidium bromide (3 μl/100 ml), at 50 V for 2 h. Bands were visualized and documented using a gel documentation system, Alpha Imager. A comparative dendrogram was made using the band pattern of ARDRA, by using NTSYS software.

DNA sequencing analysis

The bands corresponding to 16S rRNA genes were gel eluted, purified and outsourced to Eorofins Scientific, Bangalore for sequencing. Following sequencing, each sequencing data set was identified using the basic local alignment search tool (BLAST), (www.ncbi.nlm.nih.gov/BLAST). All sequences were manually aligned with closely related sequences after BLAST searches. The phylogenetic tree was constructed using the MEGA 6.0 (Tamura et al. 2013) program by the neighbour-joining method (Saitou and Nei 1987). The pairwise-deletion option was used. Bootstrap analysis of the neighbour-joining data was carried out using 1,000 resamplings. The tree was rooted using the AM181748 sequence of *Ensifer kostiense* LMG 19227 as an outgroup. The 16S rRNA gene sequences were deposited to GenBank using the Bankit submission tool, and were assigned with NCBI accession numbers KC354600 (TS2) and KC412232 (TWII).

Results and discussion

Isolation of microorganisms

Isolation and purification procedures were carried out with both water and soil samples from the hot spring of Soldhar. The temperature and pH of the sampling site was 90°C and 7.5, respectively. A total of 50 and 57 CFUs were isolated from soil and water samples, respectively, collected from the hot spring under study. Morphologically distinct colonies were picked up and pure cultures were successfully prepared for all the isolates. Pure cultures were maintained on trypton soy agar slants as well as 20 % glycerol stock preserved at -20^0C. A total of 11 strains were finally selected and characterized by morphological, physiological, and biochemical characteristics, and genetic features. All the strains developed colonies within 24 h of incubation at 70 °C, thereby indicating the fastidious nature of microorganisms in geothermal springs. The morphological features of thermophilic bacteria isolated from Soldhar hot spring are depicted in Table 1. The size of bacterial isolates varied from small (TS5, TS7 and TS8; 0.5 to 1 mm), moderate (TS2, TS3, TWI and TWII; 1–3 mm), or large (TS1 and TS6; 3–5 mm) to pinpoint (TWIII and TW1V; < 0.5 mm). White-colored bacterial colonies dominated the yellow and creamish isolates from Soldhar hot spring. All the isolates from water samples, i.e., TWI, TWII, TWIII and TWIV, exhibited serrate margins, while most of the isolates from soil samples, i.e., TS2, TS5, TS6, TS7 and TS8, exhibited entire margins, along with serrate (TS3) and lobed (TS1) margins. All the 11 bacterial isolates unanimously exhibited flat elevation and circular form. Microscopic observations revealed that the isolates were either rod shaped (TS1, TS2,

Table 1 Morphological and biochemical characteristics of bacterial isolates

Phylogenetic group based on ARDRA											
Colony/Culture	Group 1		Group 2	Group 3	Group 4					Group 5	Group 6
Characteristic	(TWIII	TWIV)	(TS3)	(TS6)	(TS5	TS7	TS8	TWI	TWII)	(TS2)	(TS1)
Size	Pinpoint	Pinpoint	Moderate	Large	Small	Small	Small	Moderate	Moderate	Moderate	Large
Pigmentation	White	White	Yellow	Cream	White	White	White	Cream	Yellow	Cream	Cream
Form	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Margin	Serrate	Serrate	Serrate	Entire	Entire	Entire	Entire	Serrate	Serrate	Entire	Lobed
Elevation	Flat	Flat	Flat	Flat	Flat	Flat	Flat	Flat	Flat	Flat	Flat
Shape	Cocci in chains	Cocci in chains	Rod	Rod	Rod	Cocci in chains	Rod	Rod	Rod	Rod	Rod
Gram Reaction	Positive	Negative	Positive	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive
Indole Test	–	–	–	–	–	–	–	–	–	–	–
Methyl-Red	–	–	–	–	–	–	–	–	–	–	–
Casein hydrolysis	+	–	+	–	+	+	–	+	+	+	–
Gelatin hydrolysis	–	–	–	–	–	–	–	–	–	–	–
Starch hydrolysis	+	+	+	+	+	+	+	+	–	+	–
Citrate utilization	–	–	–	–	–	–	–	–	+	–	+
H_2S producti	–	–	–	–	–	–	–	–	–	–	–
Urease	+	+	+	+	+	+	+	+	+	+	+
Catalase	–	–	–	–	–	–	–	–	+	+	+
Lactose	+	+	+	+	+	+	+	+	–	–	+
Xylose	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	–	–	+
Raffinose	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	–	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+

'+' and '–' indicate positive and negative results, respectively, for the test conducted

TS3, TS5, TS6, TS8, TWI and TWII) or cocci in chains (TS7, TWIII and TWIV). Most of the isolates were Gram positive, thereby outnumbering the Gram-negative bacteria (TS5 and TWIV).

The characterization of thermophilic bacteria that are able to grow at 70 °C, was done for the first time by Miquel (1888). Since then, many thermophilic and more than 20 different genera of hyperthermophilic archaea have been isolated from geothermal and hydrothermal environments (Arab et al. 2000). Our results are in consensus with those of Inan et al. (2011). They reported the isolation of nine bacterial strains from the hot spring of Turkey and all the strains were Gram positive and exhibited maximum growth at 55 °C around neutral pH. Narayan et al. (2008) also reported the dominance of Gram-positive thermophilic bacteria from the hot springs of Fiji. Sati et al. (2013) reported 25 morphologically different isolates from cold desert Himalaya; all 14 bacterial isolates were creamish, rod shaped and Gram positive. Colonization of extreme temperature (low or high) environments by a variety of microorganisms in the Himalayan region has been reported in previous studies (Sharma et al. 2009; Rinu and Pandey 2010). Particularly, many strains of the thermophilic bacteria have been found to be members of the *Bacillus* and *Clostridium* genera (Belduz et al. 2003). The dominance of species of *Bacillus* in extreme conditions is attributed to their ability to resist the environmental stresses due to their spore forming nature (Mongkolthanaruk 2012).

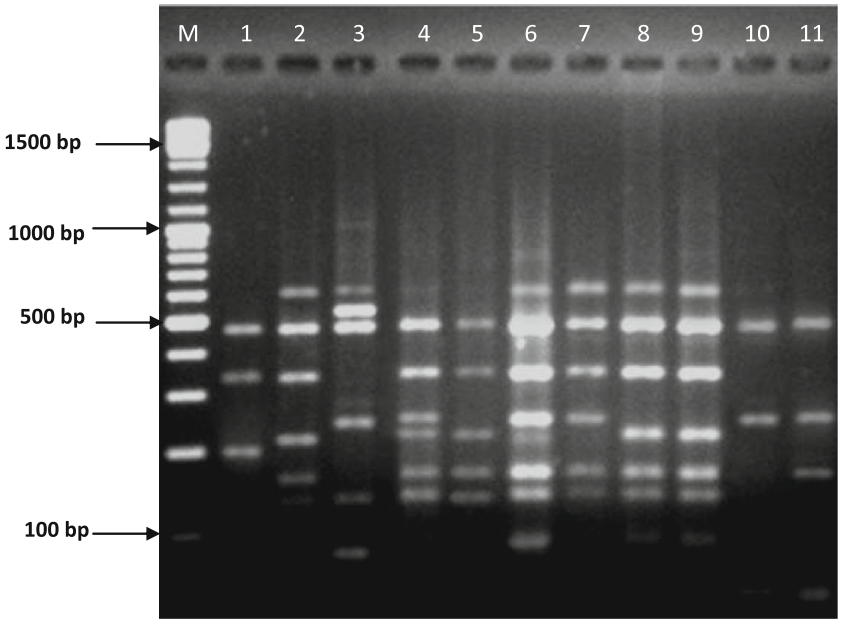

Fig. 1 ARDRA patterns of selected strains isolated from Soldhar (Tapovan) using *HaeIII* enzyme. [M-100 bp, 1-TS1, 2-TS2, 3-TS3, 4-TS5, 5-TS6, 6-TS7, 7-TS8, 8-TWI, 9- TWII, 10-TWIII, 11-TIV]

Physiological and biochemical characterization

All the isolates were found to grow over a wide temperature (20–100°C) and pH range (5–10). Inan et al. (2011) isolated nine bacterial strains from hot springs in Turkey and reported their abundant growth on media with varying pH range of 5.5-9, at temperatures of 30–65ºC.

The biochemical features of the test isolates are reported in Table 1. Except for TS1 and TWII, all other isolates hydrolyzed starch, while four out of 11 isolates, i.e., TS1, TS6, TS8 and TWIV, did not show hydrolysis of casein. Only two isolates, TS1 and TWII, exhibited citrate utilization. None of our isolates responded to gelatin hydrolysis, H_2S production, indole or methyl red test. The level of enzymatic activity

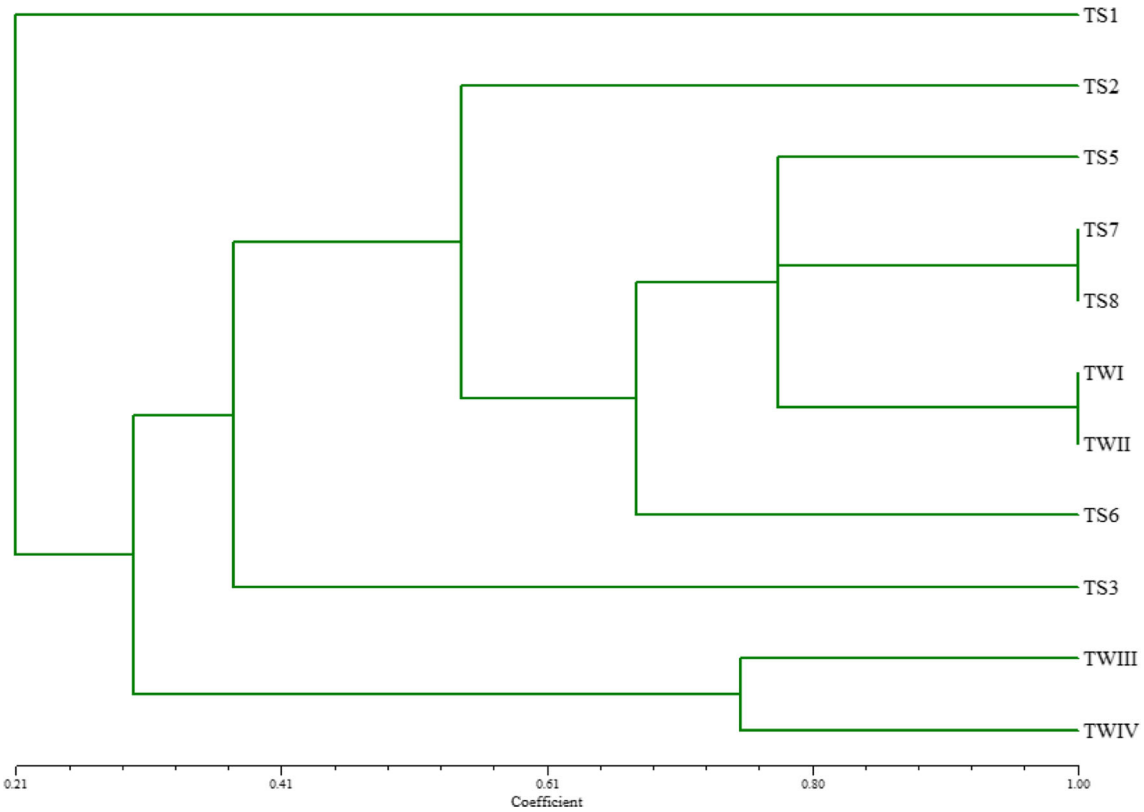

Fig. 2 Dendrogram based on ARDRA patterns of selected strains isolated from Soldhar (Tapovan) using *HaeIII* enzyme

varied in all the isolates. Less than 30 % of isolates (TS1, TS2 and TWII) showed catalase activity, while 100 % of the isolates exhibited urease activity. All of our isolates were capable of fermenting nine out of 12 sugars: xylose, maltose, fructose, dextrose, raffinose, trehalose, melibiose, arabinose and mannose. TS2 and TWII fermented all the carbohydrates except for lactose and galactose, while sucrose was fermented by all isolates except for TWI. As reported earlier, nine thermophilic bacterial isolates from the hot springs of Turkey were capable of fermenting glucose, fructose, maltose and sucrose, while more than 70 % of isolates exhibited starch hydrolysis and oxidase activity (Inan et al. 2011). Sati et al. (2013) reported the isolation of 25 morphologically different isolates from cold Himalayan desert, of which 24 isolates were positive for lipase, 19 each for protease and xylanase, 14 for amylase, 11 for pectinase, and nine for cellulase. All the thermophilic bacterial isolates from the hot springs of Fiji also exhibited amylase, lipase and gelatinase activity (Narayan et al. 2008).

16S rRNA gene sequence and ARDRA analysis

When the 16S rDNA of each of the 11 isolates was amplified by PCR, an amplification fragment of about 1,500 bp was observed. ARDRA was carried out on the amplified DNA of each sample with the restriction enzyme *Hae*III. Each pattern was compared with all the others, and this allowed eight ARDRA profiles or haplotypes to be recognized. Figure 1 represents the ARDRA pattern obtained with the restriction enzyme *Hae*III. As shown in Fig. 1, each group presented a specific banding pattern, and groups of different species were clearly differentiated.

A total of seven fragments were produced by *Hae*III on digestion (Fig. 1). Six isolates, namely, TS1, TS2, TS3, TS8, TWIII and TWIV, showed unique restriction patterns when digested with *Hae*III, while TS6, TWI and TWII showed similar restriction patterns and were placed under same group. TS5 and TS7 isolates also showed identical restriction pattern when digested with *Hae*III (Fig. 1).

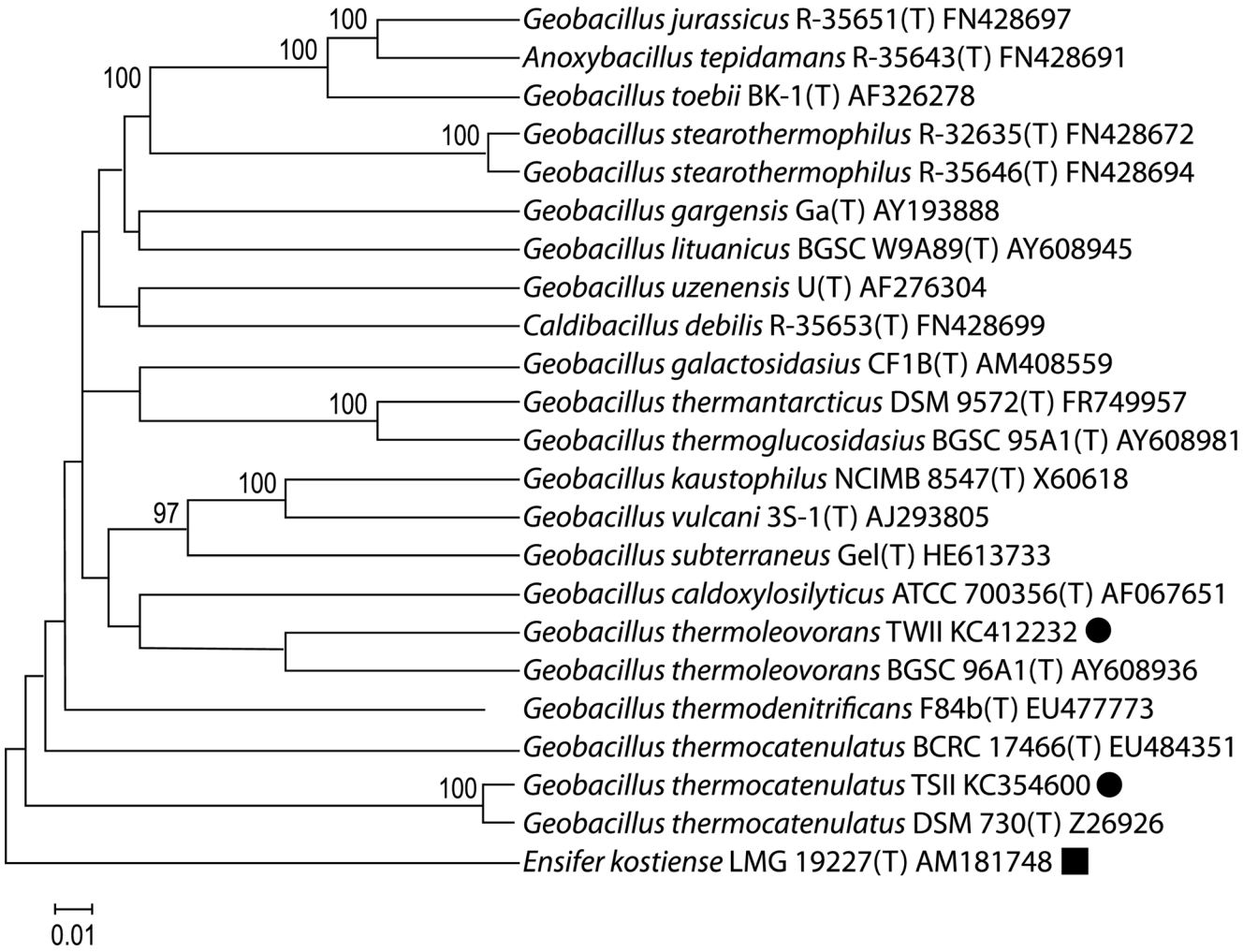

Fig. 3 Phylogenetic position of the test isolates with respect to the *Geobacillus* sp. type strains and nearest neighbors. The numbers at the nodes represent percent of bootstrap values obtained from 1,000 samplings. Only the highly significant values (higher than 70 %) are shown. The *scale bar* defines 0.01 nucleotide substitution per site. (*black circle* = Test strains, *black square* = outgroup

Based on restriction digestion by *Hae*III, six phylogenetic groups were formed (Fig. 2): TWIII and TWIV were in first group; the second group had the single isolate TS3; the third group also consisted of a single isolate, TS6; the fourth group had the maximum of five isolates, TS5, TS7, TS8, TWI and TWII; the fifth group consisted of TS2, while the isolate TS1 was the only member of sixth group as created by *Hae*III digestion. Of all the 11 isolates, only one isolate, TS1, existed as a distinct isolate of an out-group in the dendrogram produced by *Hae*III digestion.

The molecular characterization of an aerobic microbial population consisting of 146 psychrotrophic bacterial strains isolated from the Ross Sea, Antarctica) using a combination of PCR-based techniques was carried out by Michaud et al. (2004). Their analysis by *Alu*I restriction patterns of 16S rDNA grouped the 146 strains into 52 different groups (haplotypes), confirming to a high degree of inter-specific genetic diversity, suggesting that several bacterial species can adapt to the extreme environmental conditions.

The components in any of the above groups, except for the first group created by *Hae*III, (comprised of TWIII and TWIV), were not morphologically (cell and colony) similar to each other. This is in concurrence with the results of Fox et al. (1992), who showed that whereas 16S rRNA gene sequences could be used routinely to distinguish and establish relationships between genera and well-resolved species, they were not necessarily a sufficient criterion to guarantee species identity. Watve et al. (2001) also reported similar observations, where the 16S rRNA gene sequences of two Gram-positive isolates gave 0.700 and 0.735 similarity values with the 16S rRNA of Gram-negative *Pseudomonas chlororaphis* and *Acinetobacter calcoaceticus*, respectively.

Bacterial strains isolated from different sources, i.e., soil and water, and exhibiting different morphological and biochemical characters appeared to fall into the same ARDRA groups. Thus, under the present investigation, morphological markers vis-à-vis genetic markers, i.e., ARDRA analysis, indicated that there is no consistency in grouping thermophilic bacterial isolates with similar morphological characters, as phenotypically similar isolates were genetically catalogued into different clusters of a dendrogram.

We determined the partial sequences of 16S rDNAs from the two bacterial isolates belonging to different ARDRA groups and those isolated from two different samples, i.e. soil and water. The partial 16S rDNA sequences of the two isolates (TS2 and TWII) were aligned and compared to sequences of related bacteria. A phylogenetic tree was constructed using the neighbor-joining method (Fig. 3).

Analysis of the 16S rRNA gene sequences confirmed that the isolate TS2 was most similar to *Geobacillus thermocatenulatus* DSM 730 T (99.58 % similarity), while TWII shares closest homology with *Geobacillus thermoleovorans* BGSC 96A1T (100 % similarity), thereby indicating that the isolates TS2 and TWII from two different samples—soil (TS2) and water (TWII)—are members of the genus *Geobacillus*. Previously, a highly thermostable bacterium *Geobacillus stearothermophilus* was isolated from the same hot spring site by Pandey et al. (2013); it exhibited growth between 55 and 95 °C and showed clear preference for high temperature for production of cell biomass. Sharma et al. (2009) have also reported the isolation of 13 thermophilic bacterial strains belonging to *Geobacillus* spp. from hot spring sites in Garhwal Himalaya. In the present study, two bacteria previously unreported from any of the hot springs located in the Garhwal region, India, have been isolated and characterized, and their phylogenetic relationships with other bacteria of the same site has been established. The present work, therefore, extends the previous sphere of information regarding the thermophilic bacterial diversity of thermal springs in India.

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