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ISOLATION AND MOLECULAR IDENTIFICATION OF A FACULTATIVELY ANAEROBIC BACTERIUM FROM THE HOT SPRING OF AZAD KASHMIR

SANA ZAHOOR*, MUHAMMAD MOHSIN JAVED, MUHAMMAD NAUMAN AFTAB AND IKRAM-UL-HAQ

Institute of Industrial Biotechnology, GC University, Lahore, Pakistan
**Corresponding author E-mail: sanazahur@yahoo.com; Tel: +92 42 99211634; Fax: +92 42 99211634*

Abstract

A thermophilic facultatively anaerobic bacterium (TP-1) was isolated from the Tatta Pani hot spring in Azad Kashmir. The isolate had entire and slimy colonies while the cells were small rods and gram-positive. Phylogenetic analysis based on partial 16S rRNA gene sequence comparisons showed high levels of similarity of the TP-1 (> 95%) with *Geobacillus pallidus*. Optimum temperature and pH for the growth of the strain TP-1 were found to be 65°C and 7.0 respectively. TP-1 gave positive results for ortho Nitrophenyl-β-D-Galactopyranosidase (ONPG) and Gelatin hydrolysis (GEL) while other tests such as Arginine dihydrolases, Lysine decarboxlase, Ornithine decarboxylases, H_2S production, Urease, Tryptophan deaminases, Indole production, Acetoin production, Fermentation/oxidation (Glucose, Mannitol, Inositol, Sorbitol, Rhamnose, Sucrose, Melibiose, Amygdalin, Arabinose) and Citrate utilization were negative.

Introduction

Thermophiles were the first extremophile to be discovered. They are defined as groups of microorganisms which grow at a temperature above 50°C, some of them actively grow at 80°C (Madigan & Mars, 1997). Natural environments for thermophilic microorganisms are widespread on Earth's surface (Bale *et al*., 1997; Barns *et al*., 1994). These can be isolated from different environments such as deep ocean-basin cores, shallow marine hot springs, petroleum reservoirs, deep-sea hydrothermal vents and the leachate of a waste pile from a canning factory (Rahman *et al*., 2004; Bae *et al*., 2005).

For several decades, thermophilic bacteria have attracted the interest of many scientists due to their biotechnological potential in addition to scientific curiosity. It is reported that thermophilic strains produce interesting biological molecules including unusual enzymes, antibiotics, anti algal compounds, anti-cancer substances and secreted sugars (Ladenstein & Antranikian, 1998). Thermostable enzymes such as α-amylase, cellulase, α-glucosidase, α-galactosidase, β-glucosidase, β-galactosidase, protease, pullulanase and xylanase, which are stable in the range of 90-105°C, can be obtained from thermophilic organisms (Adams *et al*., 1995; Adams & Kelly, 1998).

In recent years, the use of 16S rRNA gene has been regarded as the 'gold standard' for identification and phylogenetic analysis of bacteria (Ludwig & Schleifer, 1999). The conserved nature, manageable size (1.5 Kb), high information content of rRNA gene and extensive database have resulted in the use of 16S rRNA gene as an ideal molecular chronometer in bacterial phylogeny. The 16S rRNA molecule comprises conserved region and nine variable regions (V1-V9) which provide genetic information to distinguish bacteria up to species and subspecies levels (Woese, 1987).

The numbers of hot springs occurrence are large but the knowledge on hot spring microflora is scanty (Adhikary & Sahu, 1987). Different regions of Pakistan deserve special attention to explore the vast potentialities of endemic and diverse microflora for their commercial usage. So the present study was planned to isolate and characterize thermophilic prokaryotic strains from hot spring of Azad Kashmir.

Materials and Methods

Sample collection: Water samples from different distances from the source (where the water oozes out) of the hot spring were collected separately in sterile wide mouthed thermal glass container, which kept the temperature of the samples constant, as well as in the aseptic culture tubes containing 1.0% LB (1% tryptone, 1% NaCl, 0.5% yeast extract) medium. These tubes were incubated for two hours in the same place into the water and transported to the lab for further processing (Khalil *et al*., 1998). In situ measurements of temperature, pH and EC were done to create the profile of hot springs.

Isolation of thermophilic bacteria: Thermophilic bacteria were isolated on Petri plates containing LB agar medium (1% tryptone, 1% NaCl, 0.5% yeast extract, 1.5% agar) through serial dilution of the samples. One hundred microlitre of the sample was poured over the media and incubated at 60°C while same quantity of sample was inoculated to the flask containing LB medium to isolate thermophiles (Clark *et al*., 1958; Llarch *et al*., 1997; Narayan *et al*., 2008).

Morphological and biochemical characterization: Different morphological and biochemical tests like Gram's staining, colony morphology, oxidation fermentation test and QTS-20 Tests were performed according to Wiegel & Ljungdahl (1981) and MacFaddin (2000).

Physiological characterization: In order to study the physiological behavior of the pure cultures, growth was determined at various incubation temperatures (20°C-85°C). The pH range for growth was studied by growing the cultures overnight (16 hrs) at 65±1°C in LB medium adjusted to different pH (4.0-10) separately (Anton *et al*., 2002). Turbidity was monitored at 600 nm on a double beam UV/VIS scanning spectrophotometer (Nair & Surendran, 2004).

Molecular characterization: Chromosomal DNA was isolated following protocol of Kronstad *et al*., (1983). 16S rRNA was amplified by PCR using two universal primers 9F and 1510R.

9F 5′-GAGTTTGATCCTGGCTCAG-3′
1510R 5′- GGTTACCTTGTTACGACTT-3′

PCR amplification was carried out with 1 µl (50ng) DNA as a template, 5 µl of 2.5 mM dNTPs, l µl of 10 µM of each primer, 5 µl of 25 mM magnesium chloride, 5 µl of IX PCR buffer (75 mM Tris-HCl pH 8.8 at 25°C, 20 mM $(NH_4)_2SO_4$ and 0.01% tween 20) and l µl of 2.5 units of Taq DNA polymerase in a 50 µl reaction mixture. The reaction mixture was incubated at 94°C for 5 min and then subjected to 35 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 1 min; followed by one final cycle of 72°C for 10 min. The PCR product was analysed by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. The PCR product was purified using spin prep gelmelt kit (Novagen). The purified 16S rRNA was then sequenced using 3130xl Genetic Analyzer. Highest similarities of the 16S rRNA gene sequence of TP-1 to that of other bacteria were searched using the BLAST tool of GenBank (Altschul *et al*., 1990). The 16S rRNA sequence of strain TP-1 was aligned with representative 16S rRNA sequences of related taxa using Clustal W software (Thompson *et al*., 1994). Phylogenetic tree was constructed using neighbor-joining method (Saitou & Nei, 1987) and Jukes and Cantor model (Jukes & Cantor, 1969) employing the software MEGA 4.0 (Tamura *et al*., 2007). The stability of relationships was assessed by performing bootstrap analysis of the neighbor-joining data based on 1000 resamplings.

Nucleotide sequence accession number: The 16S rRNA sequence of strain TP-1 has been deposited in the GenBank database under accession no. JQ319387.

Results

In situ measurement of pH and temperature showed that the hot spring had slightly alkaline pH i.e., 7.5 and temperature ranging from 60°C (10 feet away from the source) to 82°C (at the source). Ex situ characterization of water sample was done to determine the chemical composition of the hot spring (Table 1).

Table 1. Physical and chemical properties of water samples from Tatta Pani hot spring.

Type of study		Result
Ca^{+2} (meq/l)		1.3
Mg^{+2} (meq/l)		1.4
Na^{+} (ppm)		228
K^{+} (ppm)		3.9
CO_3^{-2} (meq/l)		4.4
HCO_3^{-} (meq/l)		15
Cl^{-} (meq/l)		0.77
TDS (g/l)		0.029
S.A.R		191.8
Temperature	Upper	82
(°C)	Lower	60
EC (mS/cm)		1.12
pH		7.5

Temperature upper = Temperature of hot spring water at the source
Temperature lower = Temperature of hot spring water at 10 feet away from source

Isolation and morphological studies: Few thermophilic bacteria were isolated from the Tatta Pani hot spring in Azad Kashmir. Isolation was performed by sampling water from surface, centre and bottom of hot spring. More than 20 colonies were isolated on LB agar plate. The bacterial culture showed good growth in LB medium and on LB agar plates (a layer of 0.8% sterile agar was poured on LB agar plate after inoculation) after incubation at 65°C, indicating the facultatively anaerobic nature of the culture. One colony showing the best growth was chosen for further study. The isolate TP-1 had small, smooth, round and slimy colonies. Morphological observation of the isolate under light microscope showed that it is rod shaped bacterium. Further analysis indicated that the isolate was gram positive (Fig. 1).

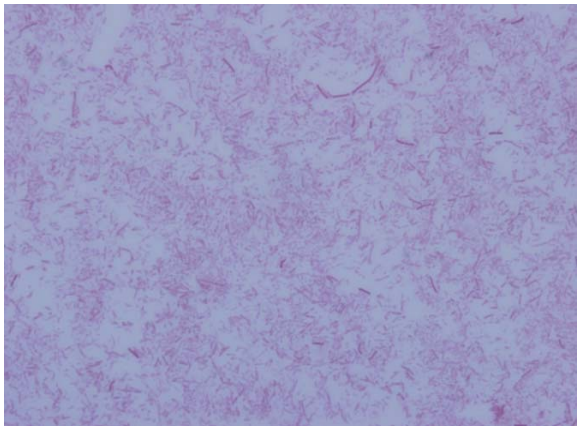

Fig. 1. Gram's staining of the strain TP-1.

Physiological and biochemical studies: Growth was observed at temperature between 20-85°C and pH ranging from 4.0-10 (Figs. 2 & 3). No growth was observed below 35°C and above 80°C. Strain TP-1 showed good growth between 55-65°C while maximum growth was obtained at 65°C and no growth was obtained at pH below 5.5 and above 8.5 while maximum growth was observed at pH 7.0.

Strain TP-1 gave positive result for gelatin hydrolysis and ortho-nitrophenyl β-D-galactopyranoside (ONPG) while gave negative results for other tests such as Arginine dihydrolases, Lysine decarboxlase, Ornithine decarboxylases, H_2S production, Urease, Tryptophan deaminases, Indole production, Acetoin production, Fermentation/oxidation (Glucose, Mannitol, Inositol, Sorbitol, Rhamnose, Sucrose, Melibiose, Amygdalin, Arabinose) and Citrate utilization.

Molecular and phylogenetic studies: Amplified 16S ribosomal RNA fragment of about 1500 bp size was obtained from strain TP-1 using 9F and 1510R universal primers (Fig. 4) and was partially sequenced (around 757 bp) using an automated DNA sequencer. The sequence was compared with 16S rRNA nucleotide sequences present in GenBank. Nucleotides BLAST search of the 16S rRNA sequences showed that the isolate had 95% identity with *Geobacillus pallidus.* A phylogenetic tree was constructed by aligning 16S rRNA sequences of 16 type strains taken from GenBank, NCBI and sequence of the TP-1 from this study (Fig. 5). The phylogenetic tree showed that the isolate was closely related to *Geobacillus pallidus.*

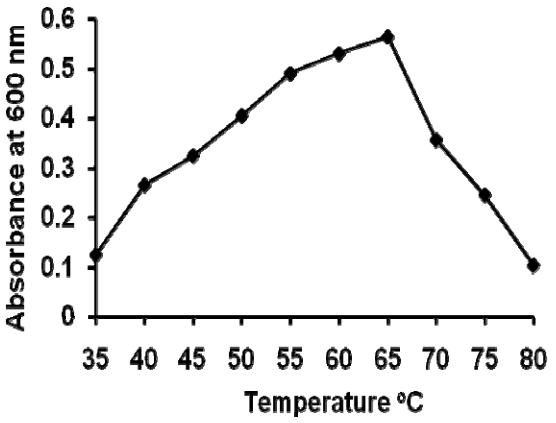

Fig. 2. Effect of temperature on the growth of strain TP-1.

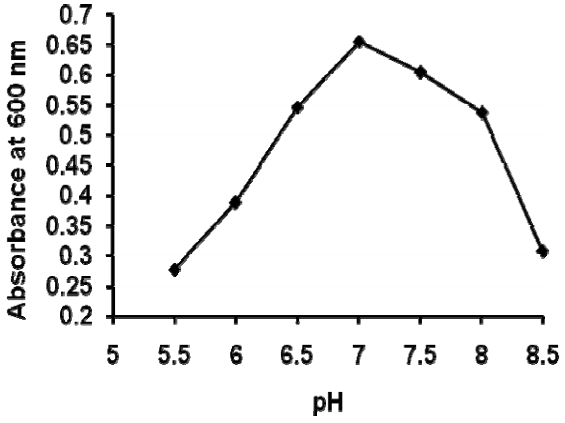

Fig. 3. Effect of pH on the growth of strainTP-1.

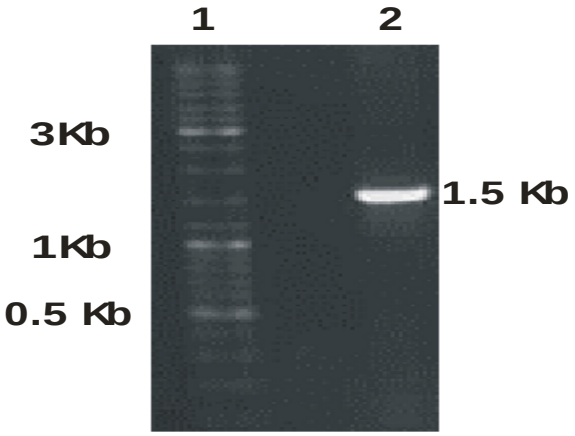

Fig. 4. PCR amplification of 16S rRNA of the strain TP-1. Lane 1 ladder, lane 2 amplified 16S rRNA.

Discussion

Our planet harbors a huge number of harsh environments that are considered as "extreme" from an anthropocentric point of view, as far as temperature, pH, osmolarity or pressure are concerned. However, these peculiar biotopes have been successfully colonized by numerous organisms, mainly extremophilic bacteria and archaea. The potential biotechnological use of thermophilic bacteria and their thermostable enzymes has led to extensive isolation studies in a wide variety of thermophilic environments. Among these environments, hot springs were explored as a potential source of thermophilic bacteria by many researchers all over the world. Narayan *et al*., (2008) isolated aerobic thermophilic bacteria from the Savasavu hot spring in Fiji. Lu *et al*., (2009) isolated thermophilic anaerobic bacteria from hot springs in Tengchong Rehai. Al-Batayneh *et al*., (2011) isolated *Geobacillus pallidus* and *Anoxybacillus flavithermus* from different hot springs in Jordan.

A fairly large number of hot springs are reported in different parts of Pakistan. Tectonic framework of the Middle East and geothermal activity in Pakistan owes its origin to the collision of the Indian Plate with the Eurasian Plate, whereby the Main Mantle Thrust (MMT) and the Main Karakoram Thrust (MKT) have been produced. The geothermal system here is the result of the collision of the Indian and Eurasian plates. Tectonic movements along these plates give rise to localization of hot springs. Tatta Pani hot springs are located on the right bank of the river Poonch, at a distance of 29 kilometers from Hajeera. They are located between the longitude 73°56´41.28" E and latitude 33°36´07.64" N, at an altitude of 1200 m.

Little information is available about the microflora of the hot springs in Pakistan. So in this study, we isolated a facultatively anaerobic thermophilic bacterium from Tatta Pani hot spring, Azad Kashmir. The isolate (TP-1) was identified by morphological analysis, biochemical tests and 16S rRNA sequence analysis. Strain TP-1 was gram-positive rod. Strain TP-1 gave positive tests for gelatin hydrolysis and ortho-nitrophenyl β-D-galactopyranoside (ONPG). The results of biochemical tests were compared with the characteristics given in Bergey's manual and they indicated that the strain TP-1 belongs to *Geobacillus.*

16S rRNA gene analysis can be used to determine the phylogenetic position of the isolate (Chang *et al*., 1998). 16S rRNA sequence analysis revealed the strain TP-1 shared 95% sequence similarity with its closest relative i.e., *Geobacillus pallidus.* But it has been demonstrated that 16S rRNA gene sequence data on an individual strain with a nearest neighbor exhibiting a similarity score of < 97% represents a new species (Petti, 2007). So sequence similarity of 95% suggests that the strain TP-1 characterizes a new species of *Geobacillus* but some more biochemical and molecular analysis is still required.

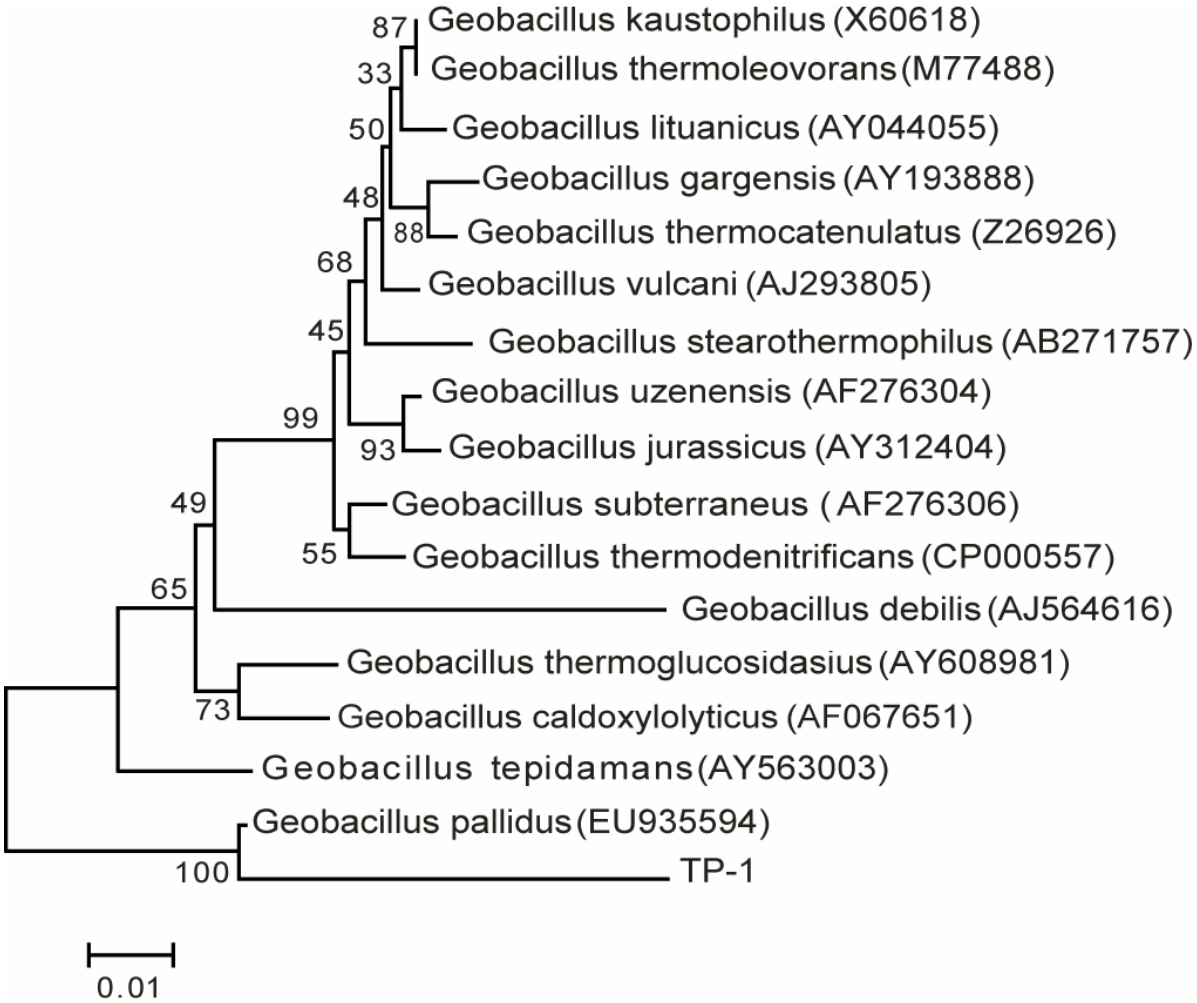

Fig. 5. Phylogenetic tree showing evolutionary relationship between isolated strain and some reference strains. The number at the branch nodes indicates bootstrap values (%) based on 1000 replications. The accession numbers are given in parentheses. The scale bar represents 0.01 nucleotide substitutes per position.

Existence of *Geobacillus pallidus* in thermal areas was also reported in many other studies. Scholz *et al*., (1987) isolated *Geobacillus pallidus* from sewage. Baharuddin *et al*., (2010) isolated *G. pallidus* from palm oil mill effluent compost and Adiguzel *et al*., (2011) confirmed the presence of *G. pallidus* in Pasinler hot spring water, Erzurum, Turkey. However, this is the first report to date demonstrating the new species of *Geobacillus* in Tatta pani hot spring, Azad Kashmir.

Investigation of different growth temperature and pH revealed that strain TP-1 gave maximum growth at 65^{o}C and pH 7.0. Further increase or decrease in temperature and pH resulted in reduced growth of the strain. Adiguzel *et al*., (2011) reported temperature of 55^{o}C and pH 7.5-8.5 for the optimum growth of *G. pallidus* isolated from Pasinler hot spring, Turkey. Al-Batayneh *et al*., (2011) optimized 60^{o}C as the growth temperature for *G. Pallidus* isolated from hot springs in Jordan.

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