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Molecular characterization of Manikaran hot spring microbial community by 16S rRNA and RAPD analysis

Bhupender Sharma*, Richa Verma, Kamal Dev, Rampa Thakur

Department of Biotechnology, Shoolini University of Biotechnology and Management Sciences, Solan, Himachal Pradesh, 173212, (INDIA)

E-mail: bhupender.sharma65@gmail.com

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ABSTRACT

The biodiversity of thermophiles in Manikaran hot spring was determined by using 16S rRNA and RAPD based methods. Thermophilic microbes were isolated from the soil and water samples collected from Manikaran hot spring, situated in Kullu district of Himachal Pradesh. The microorganisms were primarily differentiated based on the morphology, colour and texture thus purified by successive streaking on nutrient agar plates. Pure cultures were further differentiated based on Gram's staining. Seven different microbial strains were analysed by RAPD using four sets of random primers. The RAPD data was further analysed by phylogenetic analysis for genetic relatedness. Further, two strains were analyzed by PCR amplification of gene encoding 16S rRNA by using standard primers (27 F/1492 R). Amplified DNA products were sequenced on both strands, using 27 F and 1492 R primers and the quality of DNA sequences were analysed using chromatogram 3.2 to remove low quality bases (nucleotides) so as to give trimmed sequences. Trimmed sequences were assembled by using mobyle Portal online merger application to give full length 16S rDNA sequence. Merged DNA sequences were analysed by BLAST (BLASTn) program at NCBI database and identified as *Pseudomonas species* (MS1-1) and *Bacillus licheniformis* (MS1-3) with 97% similarity.

KEYWORDS

Thermophiles;
Isolation;
Gram's staining;
RAPD;
16S rRNA;
BLAST.

INTRODUCTION

Interest for this research was prompted by the fact that few studies have been conducted on thermal hot springs of India. There are two major thermal hot spring in Himachal Pradesh viz. Manikaran hot spring and Vashisht hot spring. Molecular characterization of microbial community of Manikaran and Vashisht hot springs by 16S rRNA and RAPD analysis has not been investigated. The 16S rRNA gene analysis and RAPD technique will give a more accurate view of the diversity of cultured microbes (thermophiles) in these hot springs. Exploration of hot spring microflora around the world would shed light in evolution of species and mi-

croflora residing deep inside the earth's atmosphere. Thermophiles are organisms with an optimum growth temperature of 50˚C[1,2]. Some extreme thermophiles (hyperthermophiles) require a very high temperature (80˚C to 121˚C) for growth[3,4]. The most important aspect of new isolates is to identify the microorganisms by the use of various modern biochemical and molecular biology approaches. These techniques in combination with computational biology have given new direction to evolutionary concepts and better classification. Identification is the process of characterizing organisms. In practical terms, identifying the genus and species of a prokaryote may be more important than understanding its genetic relationship to other microbes[5,6].

The sequence of rRNA molecules has been widely used for phylogenetic studies. They can be employed as a means to identify an unknown bacterium to the genus or species level[7]. rRNA sequences are popular in bacteriology because it is often easier to identify bacteria by specific nucleic acid sequences rather than by their biochemical or physiological traits. Earlier methods relied on determining the sequence of the rRNA molecule itself while newer techniques employ amplication and sequencing of rDNA, the DNA that encodes rRNA[6]. Amongst the different rRNAs, the 16S rRNA (found in 30S subunit) molecule is most useful in taxanomy because of its moderate size of approximately 1,500 nucleotides. The 5S molecule lacks the critical amount of information because of its small size (120 nucleotides), whereas the larger size of the 23S molecule (approximately 3,000 nucleotides) has made it more difficult to sequence in the past[6,8]. The prokaryotic 16S rRNA gene consists of both conserved and divergent regions[9]. Changes in these highly conserved regions occur very slowly over time and are thus useful for determining even distant relationships of diverse organisms. On the other hand, by comparing sequences in divergent regions, more recent divergence can be determined. Once the nucleotide sequence of that region has been determined, it can be compared with the 16S rDNA sequences of known organisms by searching extensive computerized databases. Thus, depending on the region compared, the sequence of rDNA can be used to assess distant as well as close relationships between organisms[6].

One of the largest genome databases is the International Nucleic Acid Sequence Data Library, often referred to as GenBank[10]. Some very useful databases are kept at the National Center for Biological Information (NCBI). The NCBI website contains a vast store of biological information, including genomic and proteomic data[11]. Basic Local Alignment Search Tool-nucleotide (BLASTn) is a widely used computer algorithm in NCBI website in searching a database[12]. BLASTn performed pairwise comparison of DNA sequences, seeking regions of local similarity rather than optimal global alignment between whole sequences[13]. It does not emphasize on the occurrence of exact sequence matches[14]. These tool aims at identifying core similarities, which are useful for later extension[15].

16S rRNA and RAPD analysis of thermophilic bacterial population present in Manikaran and Vashisht hot springs situated in Kullu district of Himachal Pradesh, diverse groups of bacteria have been identified and could be further explored for industrially important thermostable enzymes and novel secondary metabolites.

Objectives of study

1) Isolation of bacterial strains from hot spring water.
2) Phylogenetic analysis by RAPD.
3) Identification of isolated bacterial strains using 16S rRNA.

MATERIALS AND METHODS

Isolation and growth of microbes from hot spring

Soil and water samples were collected from Manikaran and Vashist hot water spring. The microflora were serially enriched three times in (2X) M9 medium (Na_2HPO_4 6g/l, KH_2PO_4 3g/l, NaCl 0.5g/l, 1mM $MgSO_4$, 100µM $CaCl_2$, NH_4Cl 2g/l, Glucose 2g/l). The soil particles were removed by centrifugation at 1000 rpm for five minutes followed by re-centrifugation at 12000 rpm for 10 minutes and cell pellet was re-suspended in M9 solution and different volumes of 100, 200, 300, 400 and 500µl were plated on nutrient agar plates and were incubated at 50˚C. The colonies thus appeared were purified by successive streaking on nutrient agar plates.

Identification and characterization of bacterial isolates

Gram's staining

Gram's staining was done for the identification of

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bacteria. Gram's staining requires the use of three reagents: Crystal violet 0.5%, iodine solution, Decolorizer (95% alcohol) and counter stain (95% Saffranine). A drop of normal saline was put onto a microscope slide with inoculating loop. The pure single colony was picked up and emulsified with normal saline to form a smear and air dried it. After drying, the thin film on the slide was fixed by passing the slide three times through the flame of a bunsen burner. The heat fixed smear was first stained with crystal violet for 1 min. After rinsing the slide, under the tap water for few seconds gently and indirectly, it was stained with iodine solution and was kept for 1 min. Then the slide was decolorized with decolorizer for 6 sec. After washing the slide under the tap water, it was stained with saffranine for 1 minute and again washed under tap water and dried on paper towels. The microbial cells were then examined under the light microscope. Gram positive cells appeared purple while Gram negative cells showed pink or red coloration. Cell morphology was also examined under the microscope.

Bacterial genomic DNA isolation

Purified bacterial colonies were inoculated overnight in 5ml sterilized nutrient broth. Next day, 1% inoculum was used to inoculate 50 ml nutrient broth medium and inoculated at 37°C until cell density reaches 4.0 ODs. The cells were collected by centrifugation at 10,000 rpm for 5 minutes. The bacterial cell pellets were re-suspended in 1ml extraction buffer (100 mM Tris HCl pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 0.07% β- mercaptoethanol, 20 mg/ml lysozyme). Afterwards 50 µl of 10 % SDS was added, mixed thoroughly and incubated for 30 minutes at 65°C. The samples were centrifuged for 20 minutes at 10,000 rpm and the supernatant was then transferred to a clean tube. RNase (1- 2 µl of 1 mg/ml stock) was added to each sample and incubated for 1 hour at 37°C. The samples were extracted three times with equal volume of Chloroform / Tris saturated phenol (1:1). DNA was precipitated by adding 1/10 sample volume of 5 M NaCl and 2.5 volumes absolute ethanol. The samples were mixed well, incubated at -20°C for 2 hours and centrifuged for 20 min at 12,000 rpm. The DNA pellet was washed with 1ml of 70% ethanol, air dried and dissolved in TE (10mM Tris HCl, 1mM EDTA) buffer and stored at -20°C until further use.

Agarose gel electrophoresis

The 1% agarose was prepared in TAE buffer. The solution was heated until it boils, the solution was cooled down to ~ 40°C and 2.5µl ethidium bromide (10 mg/ml stock) was added. The molten agarose was poured into gel casting tray and comb was placed. After the gel was solidified the comb was removed. The casting tray carrying the gel was placed into the electrophoresis tank. TAE buffer was carefully added until the buffer was 2-3 mm above the surface of the gel. 10µl of DNA samples were taken and mixed with 2µl of 6x gel 1oading dye. The samples were loaded into the wells of agarose gel. The DNA molecular weight marker was loaded to know the molecular size of the DNA samples. Electrophoresis was performed for approximately 1 h at 80 volts. The DNA bands were visualized under the UV-trans- illuminator.

DNA quantification and quality analysis: Spectrophotometric method

After isolation of genomic DNA, quantification and analysis of quality are necessary to ascertain the approximate quantity of DNA obtained and the suitability of DNA sample for further analysis. The spectrophotometer was calibrated at 260 nm as well as 280 nm by using 3 ml TE buffer. TE buffer was used as reference / blank in spectrophotometer. The 5 µl of each DNA samples were added to 2,995 µl TE buffer and mixed well. Diluted DNA samples were placed in quartz cuvette and measured the absorbance at 260 and 280 nm. The 260/280 nm was also measured.

Calculations

The ratio of absorbance at 260 to 280 nm indicates the purity of DNA samples. The ratio of absorbance of DNA samples should be in the range of 1.7 - 1.8. The presence of impurities like proteins or phenols tend to decrease this ratio and the presence of RNA contamination increases this ratio,

$A_{260}/A_{280} > 1.9$ will have RNA contamination

$A_{260}/A_{280} < 1.8$ will have Protein contamination.

The amount of DNA was quantified using the formula:

DNA concentration (µg/ml) = OD_{260} × Dilution factor × 50 µg/ml

Standard formula for quantification of nucleic acids

1 A_{260} of ds DNA = 50 µg/ml

1 A_{260} of ss oligonucleotides = 33 µg/ml

1 A_{260} of ss RNA = 40 µg/ml

RAPD (Random amplification of polymorphic DNA) analysis

DNA amplification of 7 bacterial stains was done by using four random decamer primers namely 1K, 2K, 9K and 10K. For RAPD reaction, 200 ng DNA was used as template in final volume of 20 µl. PCR mixture was prepared as described in TABLE 1. PCR master mixture components were mixed gently and centrifuged for 2-3 s. After being prepared, 18 µl of the PCR mixture was then aliquated into 0.5 ml PCR tubes, followed by addition of 2 µl genomic DNA of different bacterial stains. The tubes were placed in the PCR machine. With initial denaturation for 2 minutes at 94˚C followed by 40 cycles of 30 seconds at 94˚C (denaturation), 30 seconds at 40˚C (annealing), 2 minutes at 72˚C (elongation) and 1 cycle of final extension at 72˚C for 10 minutes.

TABLE 1 : Master mix components of PCR reaction for RAPD analysis.

Sr. No.	PCR Reagents	Reagent volume	Final concentration (in 20 µl Reaction mixture)
1	Autoclaved water	11.6 µl	-
2	10×PCR buffer	2.0 µl	1X
3	2 mM dNTPs mixture (pH 8.0)	2.0 µl	200 µM
4	Taq DNA polymerase (5 units/µl)	0.4 µl	2 U
5	Random primer (40 µM)	2.0 µl	4 µM
6	DNA template	2.0 µl	200 ng
	Total volume	20 µl	-

PCR products were either immediately analyzed by electrophoresis or stored at -20˚C until further use.

TABLE 2 : Oligoprimers sequences used for RAPD and 16 S rDNA amplification.

Primer (200µM)	Sequence Text (5'- 3')	Length	T_m	% GC
1K	CAGGCCCTTC	10	43.6	70
2K	TGCCGAGCTG	10	43.6	70
9K	CATCCCCCTG	10	43.6	70
10K	TGCGCCCTTC	10	43.6	70
27F	AGAGTTTGATCCTGGCTCAG	20	52	50
1492R	GGTTACCTTGTTACGACTT	19	47	42

Polymerase chain reaction (PCR)

PCR was performed for the amplification of 16S rDNA region by using 27F (forward primer) and 1492R (reverse primers). PCR mixture was prepared as described in TABLE 3. PCR master mixture components were mixed gently and centrifuged for 2-3 seconds. After being prepared, 18 µl of the PCR mixture was aliquated into 0.5 ml PCR tubes, followed by addition of different bacterial isolates. The tubes were placed in the PCR machine. The amplification program consisted of initial denaturation for 2 minutes at 94˚C followed by 35 cycles of 30 seconds at 94˚C (denaturation), 30 seconds at 45˚C (annealing), 2 minutes at 72˚C (elongation) and 1 cycle of final extension at 72˚C for 10 minutes.

TABLE 3 : Composition of PCR reaction for the amplification of 16S rDNA.

Sr. No.	PCR Reagents	Reagent volume	Final concentration (in 20 µl reaction mixture)
1	Autoclaved water	11.6 µl	-
2	10×PCR buffer	2.0 µl	1X
3	2 mM dNTPs mixture (pH 8.0)	2.0 µl	200 µM
4	20 µM forward primer	1.0 µl	1 µM
5	20 µM reverse primer	1.0 µl	1 µM
6	Thermostable DNA polymerase (5 units/µl)	0.4 µl	2 U
7	DNA template	2.0 µl	200 ng
8	Total volume	20µl	-

For each DNA sample, reactions were performed in triplicates. Subsequently, the PCR amplicons (5µl+1µl loading dye) were analyzed by gel electrophoresis through 1% agarose gel at 70V for 60 minutes.

Purification of PCR products by Axyprep PCR clean up kit

Three volume of solubilization buffer (150µl of DE-A) was added to the each PCR sample (50µl). Followed by this, 125µl binding buffer (DE-B) containing 50µl isopropanol was added. The miniprep columns were placed onto 2 ml microfuge tubes and sample mixtures were loaded on to the spin columns and centrifuged at 12,000 g for 1 minute. The filtrate was discarded and the columns were replaced back on the

microfuge tubes. The columns were washed with 500µl of wash buffer by centrifuging at 12,000 g for 30 seconds and the filtrate was discarded. The columns were further washed with 700 µl desalting buffer (W2) by centrifuging at 12,000 g for 1 minute and the flow through / filtrate was discarded. The miniprep columns were replaced back to microfuge tubes and centrifuged at 12,000 g for 1 minute to remove the remaining wash buffers from the columns. The column was placed on to a fresh/clean 1.5 ml microfuge tube. To elute the DNA, 25-30µl of eluent or deionized water was added to the center of the membrane and allowed it to stand for one minute at room temperature and centrifuge at 12,000 g for one minute. The purified DNA samples were stored at -20°C until mailed for DNA sequencing with 27F and 1492R primers.

DNA sequencing and analysis

DNA Sequencing was performed by using 27 F as forward primer and 1492 R as reverse primers. The quality of DNA sequences were analysed by using chromatogram explorer version 3.1.1 to remove low quality bases (nucleotides) so as to give trimmed sequences. High quality sequences with quality value above 45 were used for further analysis. Sequences with low QV were discarded and/or removed. Good quality and trimmed sequences were assembled by using mobyle Portal (http://mobyle.pasteur.fr/cgi-bin/portal.py#welcome) online merger application to give full length 16S rDNA sequence.

Analyses of DNA sequences

BLASTn analysis

The (16S rDNA sequences) merged sequences were analyzed by BLAST at NCBI database using nucleotide BLAST (BLASTn) program. The hits with highest identities and closest homology were selected.

Sequence match

The 16S rDNA sequence of MS1-1 and MS1-3 were submitted as query to Ribosomal Database Project-II (RDP) Release 10 (http://rdp.cme.msu.edu/classifier/classifier.jsp). Sequence Match program was used to identify MS1-1 and MS1-3.

Sequence match is a crude but fast way of measuring between query sequence and unaligned sequences in the Ribosomal Database Project-II Release 10 (RDP) database (RDP documentation). It was used to identify query instead of homology and similarity search because there is no direct relationship between sequence match and the sequence similarity. Sequence match does not use mismatched for its calculation. Hence, neither rRNA nor rDNA similarity values can be used to detect fine differences between highly related species[14].

RESULTS AND DISCUSSION

Isolation and purification of bacterial isolates

Twenty one different bacterial strains were isolated and purified from Manikaran and Vashisht hot water springs of Kullu district of Himachal Pradesh. Bacterial stains were isolated from soil and water samples. Culturing was carried out on nutrient agar plates. The colonies thus appeared were purified by successive streaking on nutrient agar plates (Figure 1).

Gram's staining and morphology

Isolated bacterial strains were subjected to Gram staining and observed under light microscope. TABLE 4 shows the Gram's staining and their morphology as observed.

Genomic DNA isolation

Bacterial genomic DNA was isolated from twenty one different bacterial strains. Isolated genomic DNA was analyzed by electrophoresis on 1 % agarose gel. 10µl DNA sample and 2 µl (6X) loading dye was loaded in the well. Electrophoresis was performed for approximately 50 min at 80 volts. The DNA bands were visualized under UV-trans-illuminator and pictures were taken by using gel documentation and shown in Figure 2.

DNA quantification and quality analysis: Spectrophotometric method

DNA can be quantified by using the formula

DNA concentration (µg/ml) = OD_{260} × Dilution factor × 50 µg/ml

Here we use 2995µl TE (Tris-EDTA buffer)
So dilution factor = 600

DNA concentration (µg/ml) = OD_{260} × 600 × 50 µg/ml

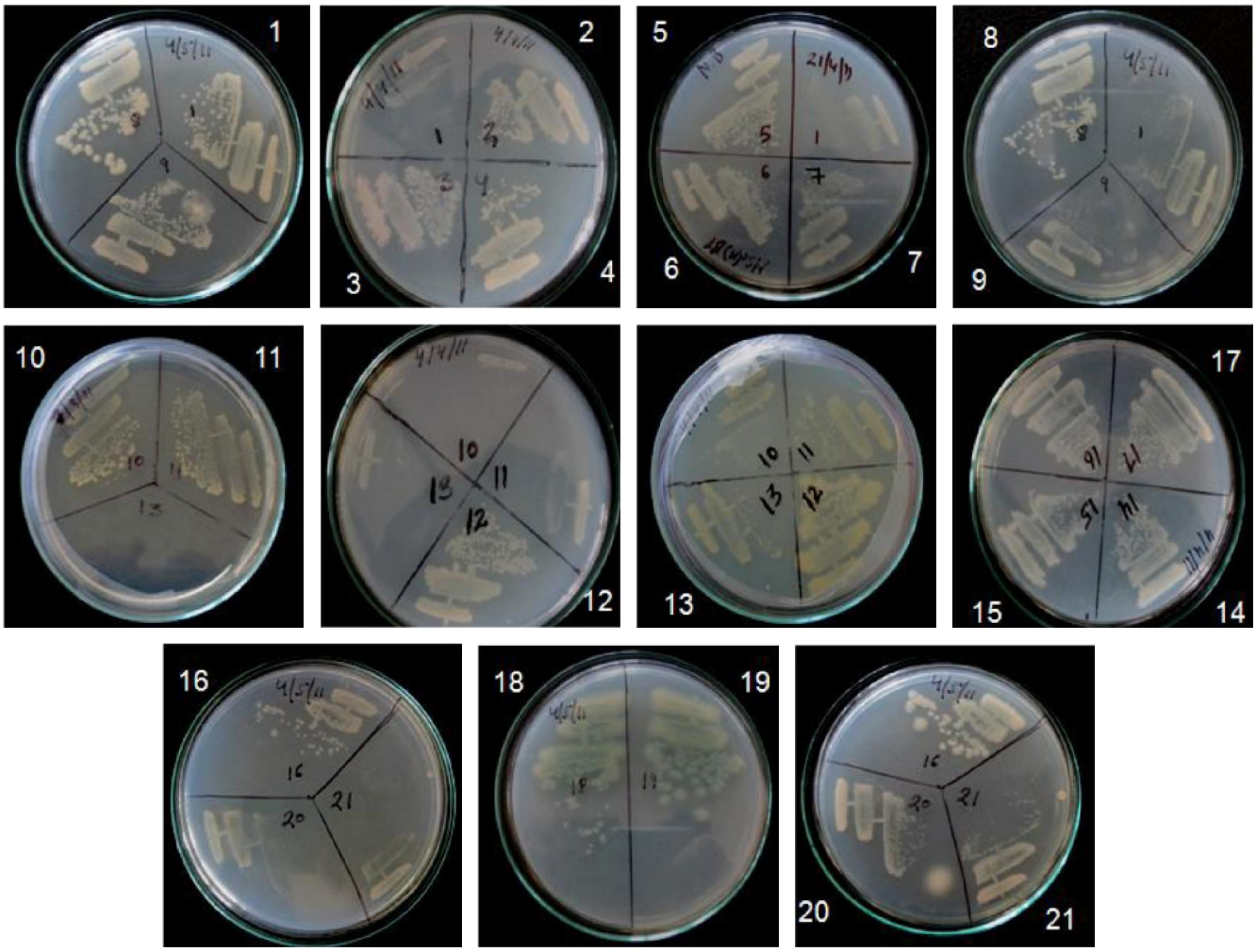

Figure 1 : Streaking of purified bacterial isolates. Bacterial strains were streaked on LB medium and incubated for 12 h at 37°C. Strains numbers are as indicated.

TABLE 4 : Gram's staining and growth temperature of bacterial strains.

Sr. No.	Sample codes	Maximum growth temperature	Gram's staining and morphology	Sr. No.	Sample codes	Maximum growth temperature	Gram's staining and morphology
1	VW-2	50°C	+ve bacilli	12	MS1-2	50°C	-ve bacilli
2	VW/2	50°C	+ve cocci	13	MS1-1	50°C	-ve bacilli
3	MS1-3	50°C	+ve bacilli	14	MS1-4	50°C	+ve cocci
4	MS2/1	50°C	-ve cocci	15	MS1-4.1	50°C	+ve cocci
5	MS2/2	50°C	-ve cocci	16	MS1-4.2	50°C	+ve cocci
6	VW/1	50°C	-ve cocci	17	MS1-4.3	50°C	-ve cocci
7	MS1/2	50°C	-ve cocci	18	MS1-5	50°C	-ve cocci
8	Pf	50°C	-ve bacilli	19	MS1-5.1	50°C	-ve cocci
9	MS1/1	50°C	-ve bacilli	20	MS1-6.1	50°C	+ve bacilli
10	VW-1	50°C	-ve bacilli	21	MS1-6	50°C	+ve bacilli
11	MS2-1	50°C	-ve bacilli				

VW; Vashisht hot spring samples and MS; Manikaran hot spring samples.

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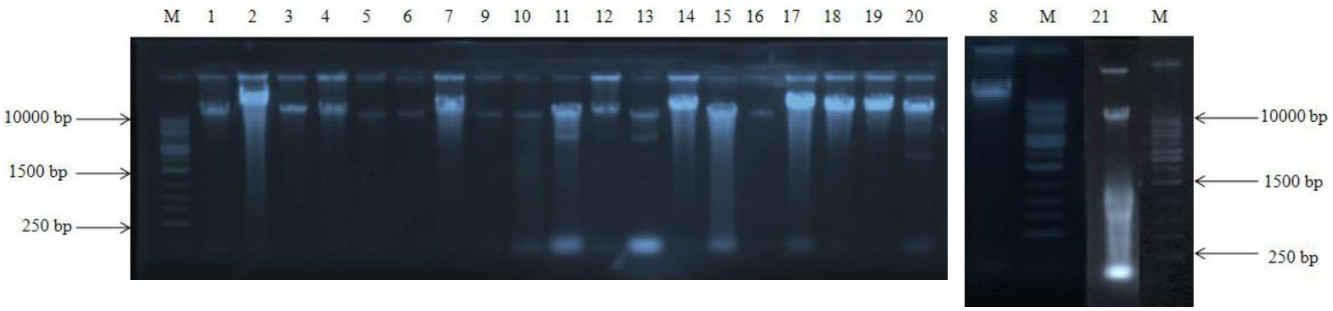


Lanes: (M) 1kb DNA ladder; (1) VW-2; (2) VW/2; (3) MS1-3; (4) MS2/1; (5) MS2/2; (6) VW/1; (7) MS1/2; (9) MS1/1; (10) VW-1; (11) MS2-1; (12) MS1-2; (13) MS1-1; (14) MS1-4; (15) MS1-4.1; (16) MS1-4.2; (17) MS1-4.3; (18) MS1-5; (19) MS1-5.1; (20) MS1-6.1; (8) Pf; (21) MS1-6.

Figure 2 : Genomic DNA of different bacterial isolates.

TABLE 5 : Spectrophotometric method for DNA quantification.

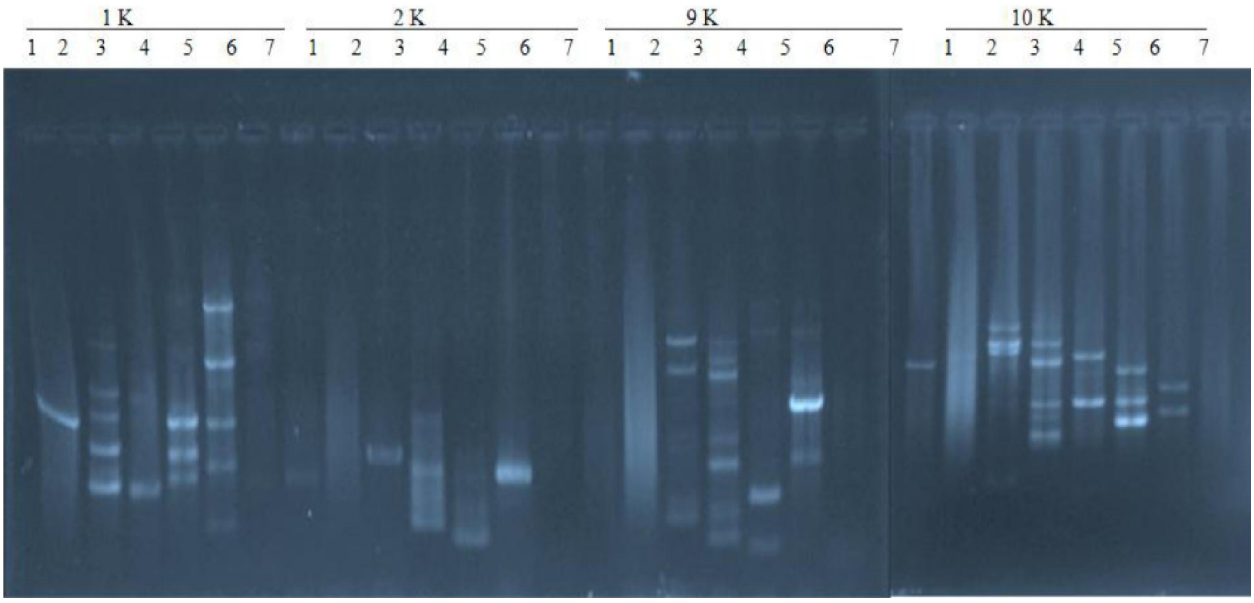
Sr. No	Sample code(s)	A260 nm	A280 nm	A260/280 nm	DNA concentration (µg/ml)	DNA concentration (µg/µl)
1	VW-2	0.023	0.013	1.77	690	0.690
2	VW/2	0.025	0.014	1.79	750	0.750
3	MS1-3	0.027	0.015	1.80	810	0.810
4	MS2/1	0.020	0.011	1.82	600	0.600
5	MS2/2	0.009	0.005	1.80	270	0.270
6	VW/1	0.007	0.004	1.75	210	0.210
7	MS1/2	0.035	0.020	1.75	1050	1.05
9	MS1/1	0.001	0.001	1.00	30	0.030
10	VW-1	0.051	0.027	1.89	1530	1.53
11	MS2-1	0.078	0.043	1.81	2340	2.34
12	MS1-2	0.011	0.006	1.83	330	0.33
13	MS1-1	0.120	0.066	1.82	3600	3.6
14	MS1-4	0.022	0.012	1.83	660	0.66
15	MS1-4.1	0.069	0.040	1.72	2070	2.07
16	MS1-4.2	0.142	0.116	1.23	4260	4.26
17	MS1-4.3	0.068	0.040	1.70	2040	2.04
18	MS1-5	0.125	0.070	1.78	3750	3.75
19	MS1-5.1	0.053	0.030	1.77	1590	1.59
20	MS1-6.1	0.070	0.038	1.84	2100	2.1
21	MS1-6	0.078	0.042	1.86	2340	2.34

RAPD analysis

Amplified PCR products were analyzed by electrophoresis on 1 % agrose gel. 10µl DNA sample was mixed with 2µl of 6 X loading dye and loaded into the wells of agarose gel starting from the second well. DNA molecular weight marker (3µl of 100 ng/ µl) was loaded into the first well. Electrophoresis was performed for approximately 90 min at 60 volts. The DNA bands were visualized under UV trans-illuminator and pictures were taken by using gel documentation and shown in Figure 3.

Similarity matrix and cluster analysis

The RAPD fragments obtained after the amplification of genomic DNA were scored for their presence (1) and absence (0) of amplicons for each sample. The data matrix was analyzed with MSVP 3.2 software. The data of similarity coefficients generated were used to construct the dendrogram indicating the genetic relatedness or differentiation using unweighted paired group method of arithmetic averages (UPGMA) algorithm.



Lanes: (1) VW/2; (2) MS1-3; (3) MS2/1; (4) VW/1; (5) MS1-2; (6) MS1-1; (7) MS2-1.
RAPD pattern by random primers 1K (CAGGCCCTTC), 2K (TGCCGAGCTG), 9K (CATCCCCCTG), 10 K (TGCGCCCTTC).

Figure 3 : RAPD patterns of bacterial strains with random primers (1K, 2K, 9K and 10K).

TABLE 6 : RAPD data matrix for RAPD fragments with random primers (1K, 2K, 9K and 10K).

RAPD Primers	Sr. No.	Sample codes							RAPD Primers	Sr. No.	Sample codes						
		VW/2	MS1-3	MS2/1	VW/1	MS1-2	MS1-1	MS2-1			VW/2	MS1-3	MS2/1	VW/1	MS1-2	MS1-1	MS2-1
	1	0	0	0	0	1	0	0	9 K	5	0	0	1	0	0	0	0
	2	0	1	0	0	0	0	0		6	0	0	0	0	1	0	0
	3	0	0	0	0	1	0	0		7	0	0	1	0	0	0	0
	4	0	1	0	0	0	0	0		8	0	0	1	0	1	0	0
	5	1	1	0	0	0	0	0		9	0	0	0	1	0	0	0
1K	6	0	0	0	1	1	0	0		10	0	1	0	0	0	0	0
	7	0	1	0	0	0	0	0		11	0	1	1	0	0	0	0
	8	0	0	0	1	0	0	0		12	0	0	1	0	0	0	0
	9	0	0	0	0	1	0	0		13	0	0	0	1	0	0	0
	10	0	0	0	1	0	0	0		1	0	1	0	0	0	0	0
	11	0	1	1	0	0	0	0		2	0	1	1	0	0	0	0
	1	0	0	1	0	0	0	0		3	0	1	0	0	0	0	0
	2	0	1	0	0	0	0	0		4	0	0	0	1	0	0	0
2 K	3	0	0	1	0	1	0	0		5	0	0	1	0	0	0	0
	4	0	0	1	0	0	0	0	10 K	6	0	0	0	0	1	0	0
	5	0	0	0	1	0	0	0		7	0	0	0	0	0	1	0
9 K	1	0	0	0	0	1	1	0		8	0	0	1	1	1	0	0
	2	0	1	1	0	0	0	0		9	0	0	0	0	0	1	0
	3	0	0	1	0	0	0	0		10	0	0	0	0	1	0	0
	4	0	1	0	0	0	0	1		11	0	0	1	0	0	0	0

TABLE 7 : Jaccard's similarity analysis.

Samples	VW/2	MS1-3	MS2/1	VW/1	MS1-2	MS1-1	MS2-1
VW/2	1.000						
MS1-3	0.077	1.000					
MS2/1	0.000	0.167	1.000				
VW/1	0.000	0.000	0.045	1.000			
MS1-2	0.000	0.000	0.130	0.118	1.000		
MS1-1	0.000	0.000	0.000	0.000	0.077	1.000	
MS2-1	0.000	0.077	0.000	0.000	0.000	0.000	1.000

PCR analysis

The 16S rRNA gene was amplified through PCR reaction using specific 16S rRNA primers and the PCR reaction products were separated on 1% agarose gel. The DNA bands were visualized under UV trans-illuminator and pictures were taken by using gel documentation and shown in Figure 5. For the purpose of

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sequencing, PCR products were further purified using Axyprep PCR clean up kit and analyzed by gel electrophoresis as shown in Figure 6.

Purification of PCR products by Axyprep PCR clean up kit

PCR products were subjected to purification by using Axyprep PCR clean up kit and analyzed by agarose gel electrophoresis.

Assembly of full length 16S rDNA sequence

The DNA sequences obtained by DNA sequencing were analyzed by chromatogram explorer version 3.1.1 to remove low quality bases (nucleotides) so as to get trimmed sequences and these trimmed sequences were assembled by using mobyle Portal online merger application. The 16S rRNA gene sequence of MS1-1 and MS1-3 is shown in Appendix 3.

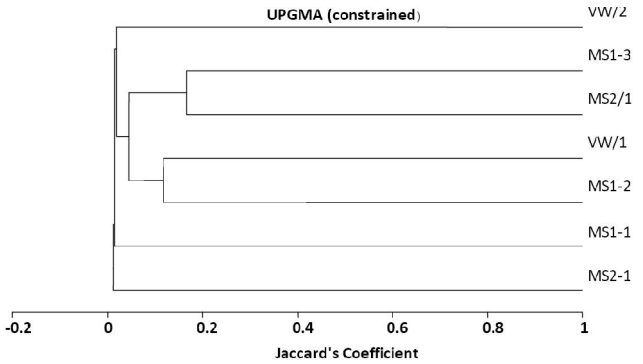
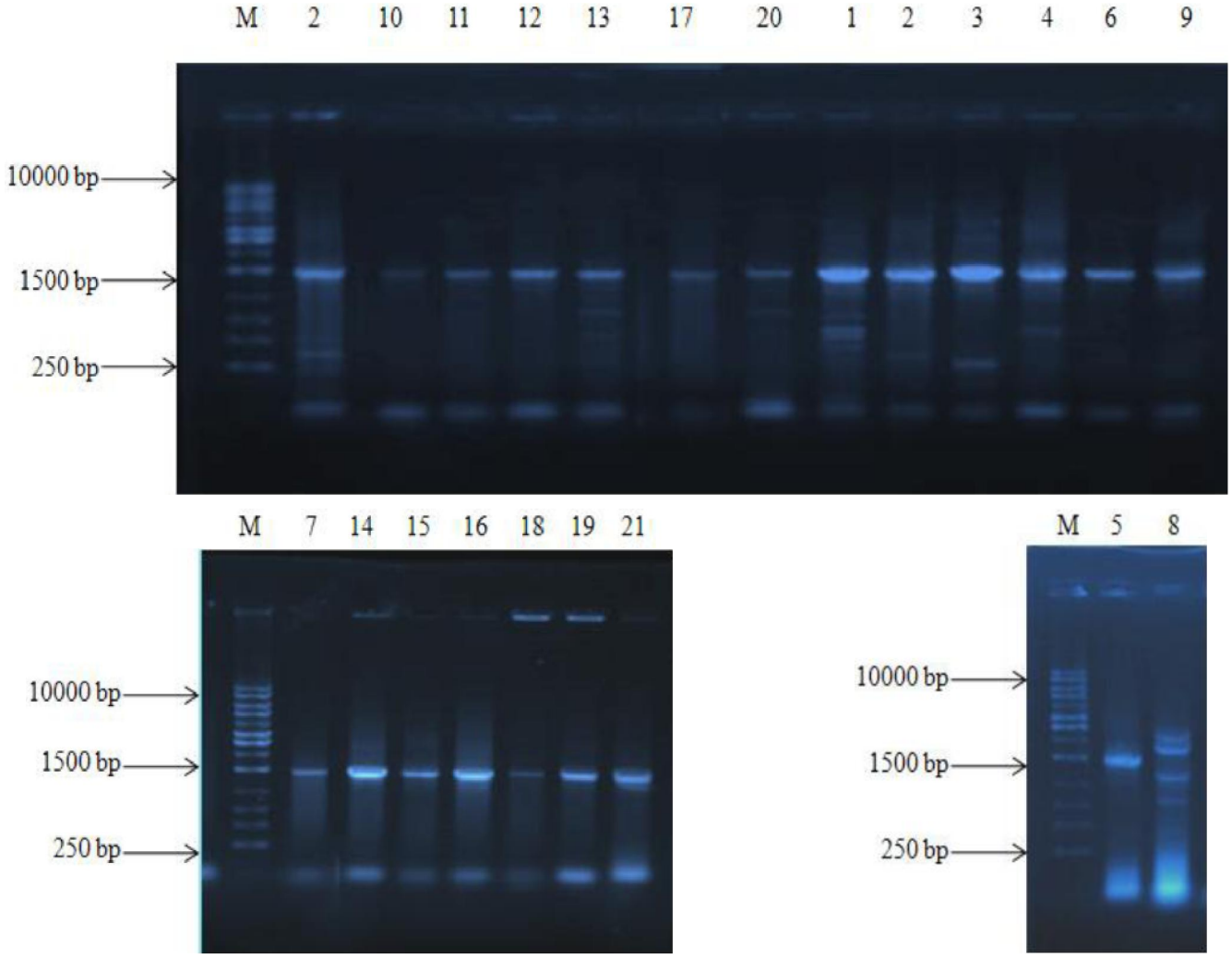


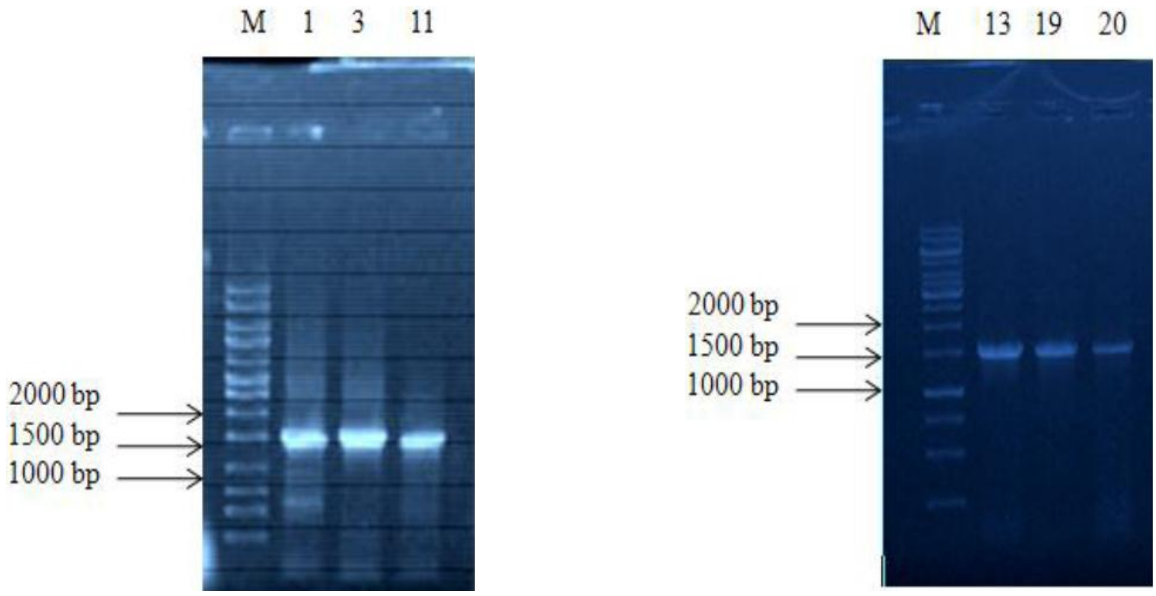
Figure 4 : UPGMA dendrogram showing genetic relatedness amongst Manikaran and Vashisht hot water springs.



Lanes: (M) 1kb DNA ladder; (1) VW-2; (2) VW/2; (3) MS1-3; (4) MS2/1; (5) MS2/2; (6) VW/1; (7) MS1/2; (8) Pf; (9) MS1/1; (10) VW-1; (11) MS2-1; (12) MS1-2; (13) MS1-1; (14) MS1-4; (15) MS1-4.1; (16) MS1-4.2; (17) MS1-4.3; (18) MS1-5; (19) MS1-5.1; (20) MS1-6.1; (21) MS1-6

PCR product less than 250 bps indicates primer dimer.

Figure 5 : Amplification of gene encoding 16S rRNA.



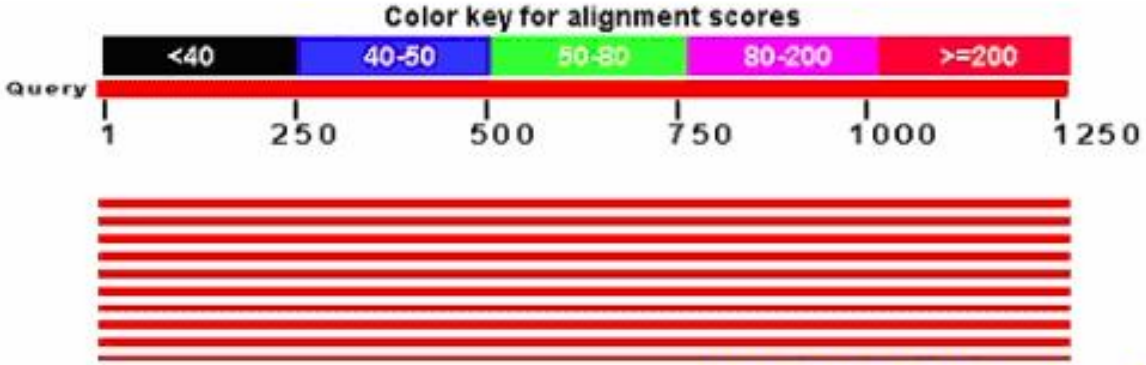
Lanes: (M) 1kb DNA ladder; (1) VW-2; (3) MS1-3; (11) MS2-1; (M2) 1 kb ladder; (13) MS1-1; (19) MS1-5.1; (20) MS1-6.1.

Figure 6 : Purification of PCR products.

Identification of MS1-1 and MS1-3

Homology and similarity search of 16S rRNA gene of bacterium MS1-1 and MS1-3

BLASTn performed pairwise comparison of DNA sequences, seeking regions of local similarity rather than optimal global alignment between whole sequences. Sequences were aligned to determine homology of the query sequence (MS1-1 / MS1-3) against sequences in genomic DNA databases (GenBank, EMBL, DDBJ, PDB) in NCBI website. The results of local alignment are shown in Figure 7 (MS1-1) and Figure 8 (MS1-3).



Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GU198101.1	Pseudomonas fluorescens strain LMG 1244 16S ribosomal RNA gene, partial sequence	2375	2375	100%	0.0	97%
NR_024928.1	Pseudomonas gessardii strain CIP 105469 16S ribosomal RNA, partial sequence >gb|AF074384.1|AF074384 Pseudomonas gessardii 16S ribosomal RNA gene, complete sequence	2375	2375	100%	0.0	97%
HM561499.2	Uncultured Pseudomonas sp. clone Dn32 16S ribosomal RNA gene, partial sequence	2374	2374	100%	0.0	97%

Figure 7 : Blast page of MS1-1 showing the first three hits with 97% DNA sequence identity.

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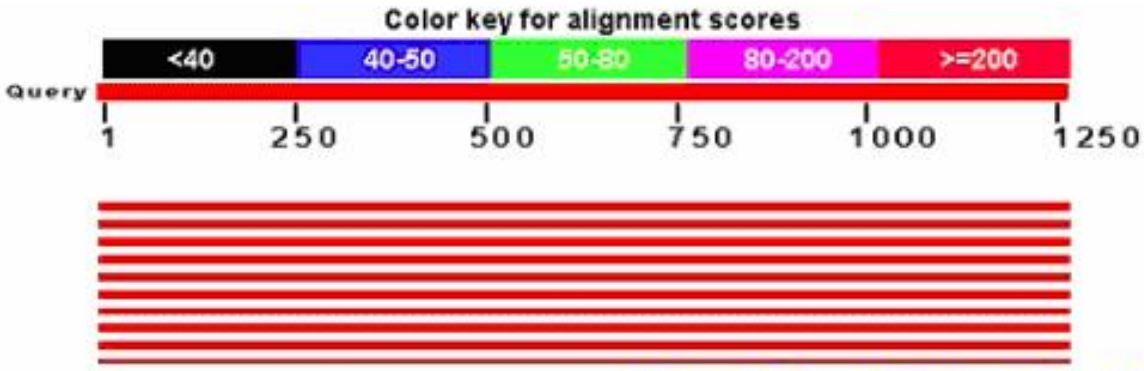

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JF796144.1	Bacillus sp. L1(2011) 16S ribosomal RNA gene, partial sequence	2274	2274	100%	0.0	97%
JF769746.1	Bacillus licheniformis strain TD4 16S ribosomal RNA gene, partial sequence	2274	2274	100%	0.0	97%
JF496512.1	Bacillus licheniformis strain WAS3-5 16S ribosomal RNA gene, partial sequence	2274	2274	100%	0.0	97%

Figure 8 : Blast page of MS1-3 showing first three hits with 97% DNA sequence identity.

Figure 7 shows a blast page for MS1-1 strain. From the BLAST result, the closest sequence matches to this clone were three; *Pseudomonas fluorescens* strain LMG 1244, *Pseudomonas gessardii* strain CIP 105469 and Uncultured *Pseudomonas sp.* clone Dn32, all of which had sequence identities of 97% to MS1-1. Therefore MS1-1 strain could be a *Pseudomonas* species. Figure 8 shows a blast pages for MS1-3 strain. From the blast result, the closest sequence matches to this clone were three; *Bacillus sp*. L1, *Bacillus licheniformis* strain TD4 and *Bacillus licheniformis* strain WAS3-5, all of which had sequence identities of 97% to MS1-3. Therefore MS1-3 strain could be a *Bacillus* species.

Query Sequence: MS1-1, 1318 unique oligos

Lineage:

no rank: Root (0/20/973768) (selected/match/total RDP sequences)
domain Bacteria (0/20/962279)
phylum "Proteobacteria" (0/20/252218)
class Gammaproteobacteria (0/20/118043)
order Pseudomonadales (0/20/45658)
family Pseudomonadaceae (0/20/20805)
genus Pseudomonas (0/20/20420)

S000126707	0.912	1211	Pseudomonas sp. IrT-R5M2-32; AJ291841
S000127323	0.906	1218	Pseudomonas sp. IrT-R5M2-56; AJ291839
S000133653	0.903	1228	Pseudomonas sp. IrT-R5M2-58; AJ291840
S000344280	0.904	1271	uncultured bacterium; ckncm328-B6-64; AF376232
S000416739	0.916	1219	uncultured gamma proteobacterium GR-296.I-27; GR-296.I.
S000435188	0.905	1286	Pseudomonas fluorescens; 302; AY092072
S000532142	0.904	1244	Pseudomonas sp. ps4-14; AY303292
S000532143	0.901	1248	Pseudomonas sp. ps4-16; AY303293
S000532147	0.911	1248	Pseudomonas sp. ps4-22; AY303297

S000532151	0.901	1249	Pseudomonas sp. ps4-29; AY303301
S000825331	0.903	1168	Bacillus sp. NY27; EF458318
S001241632	0.912	1277	Pseudomonas sp. XH1; FJ424509
S001263769	0.908	1258	Pseudomonas sp. 104; FJ013262
S001551285	0.901	1277	Pseudomonas sp. 33D; GQ174493
S002165084	0.906	1299	Pseudomonas sp. DSP-R33; AB547164
S002165086	0.902	1305	Pseudomonas sp. DSP-S3; AB547166
S002230069	0.915	1277	Pseudomonas sp. a001-37; HM468063
S002230102	0.916	1281	Pseudomonas sp. a111-62; HM468096
S002235531	0.908	1265	bacterium A66; HQ332149
S002235533	0.908	1262	bacterium A119; HQ332151

Figure 9 : Result of sequence match for bacterial stain MS1-1.

Query Sequence: 27F_MS1-3, 1266 unique oligos

Lineage:

no rank: Root (0/20/973768) (selected/match/total RDP sequences)
domain Bacteria (0/20/962279)
phylum "Firmicutes" (0/20/383115)
class "Bacilli" (0/20/257815)
order Bacillales (0/20/168991)
family Bacillaceae (0/20/19213)
genus Bacillus (0/20/14692)

S000345156	0.877	1421	Bacillus licheniformis; AB111114; C16; AF397062
S000358759	0.877	1461	Bacillus licheniformis; C16; AY479984
S000365541	0.877	1456	Bacillus sp. TUT1217; AB188216
S000392549	0.877	1427	Bacillus licheniformis; Mo1; AF372616
S000467898	0.877	1392	Bacillus licheniformis; CICC10181; AY842871
S000538901	0.877	1405	Bacillus licheniformis; CICC 10104; DQ082997
S000615411	0.877	1409	Bacillus licheniformis; ACO1; DQ228696
S000647676	0.877	1421	Bacillus licheniformis; K19; DQ351932
S000843501	0.877	1442	Bacillus sp. J24; EF471917
S000870628	0.877	1349	Bacillus licheniformis; DstI-4; AB305269
S000870631	0.877	1340	Bacillus licheniformis; YS2-1; AB305272
S000870632	0.877	1340	Bacillus licheniformis; YS2-2; AB305273
S000870634	0.877	1340	Bacillus licheniformis; YS2-4; AB305275
S000902654	0.877	1322	Bacillus licheniformis; R-J-101; EU016215
S001093930	0.877	1326	Bacillus sp. US2 HS-2008; AM950310
S001093934	0.877	1326	Bacillus sp. US4 HS-2008; AM950314
S001152805	0.877	1342	Bacillus sp. LY2; EU073121
S001155470	0.877	1361	Bacillus sp. BgR2; EU864533
S001550910	0.878	1383	Bacillus licheniformis; CGMCC 2876; GQ148817
S002234899	0.879	1331	Bacillus licheniformis; SAT2-11; HQ236037

Figure 10 : Result of sequence match for bacterial stain MS1-3.

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Sequence match

There were 1318 unique oligomers in MS1-1 query sequence and 1266 unique oligomers in MS1-3 query sequence. MS1-1 posed 91.6 % similarity with that of *Pseudomonas sp.* a111-62; (HM468096), whereas MS1-3 posed 87.9 % similarity with that of *Bacillus licheniformis*; SAT2-11; HQ236037. As proposed by Stackebrandt and Goebel (1994), a prokaryotic 16S rRNA with less than 97 % identical to any other sequence should be considered as a new species[16]. Thus this Sequence Match value (91.6 %) and (87.9%) may be new species of *Pseudomonas* and *Bacillus* respectively.

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