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ORIGINAL PAPER

Molecular characterization of thermophilic bacteria isolated from Van City Ercis Town Hasanabdal hot spring

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Abstract

The study was carried out in order to determine the phenotypic and genotypic characterization of thermophilic bacteria isolated from Van city Ercis town Hasanabdal hot spring water, Turkey. Fatty Acid, rep PCR profiling methods, and 16S rDNA sequence data were used for the molecular characterization of thermophilic bacteria. Totally 9 different bacterial isolates were selected based on morphological, physiological and biochemical tests. These strains were further characterized by molecular tests including fatty acid and rep PCR profiles, and 16S rDNA sequence. As the Fatty Acid profiles were examined, it was determined that for P13 and P9 isolates, iso-C15:0, anteiso-C15:0, iso-C17:0 and anteiso-C17:0 fatty acids are the major fatty acids. For P22, P86, P56, P60, P59 and P4 isolates, the major fatty acids are C15:0, iso-C16:0, and iso-C17:0 fatty acids, and the major fatty acids for P14, P35 isolates are iso-C15:0 and anteiso-C15: 0 fatty acids. These bacterial strains that differ in terms of fatty acid profiles were analyzed by REP and BOX-PCR, which are a genomic fingerprint analysis method. For the strains, which exhibited differences with respect to each other at the end of this analysis, 16S rDNA analyses were performed. As a result of 16S rDNA analyses, it was observed that six of these isolates were identified as Geobacillus pallidus and remaining four strains were Bacillus licheniformis, B. pumilus, Brevibacillus brevis and Br. borstelensis species respectively.

Keywords: Thermophile bacteria; FAMEs; REP-PCR; BOX-PCR; 16S rRNA sequencing.

Introduction

The discovery of life at elevated temperatures and the isolation of *Thermus aquaticus* from Yellowstone National Park had been a great step in biotechnology. Following this, PCR technology appeared aiming especially the replication of DNA under *in vitro* conditions. Particularly, *Thermus aquaticus* success in commercial areas led scientists to the

researches of the isolation and identification of microorganisms from geothermal sources of which microbial constitution has not been determined, yet [1,2].

The first research about the characterization of thermophilic bacteria which were forming aerobic spores and able to grow at 70°C was done by Miquel in 1888 [3]. Since then, as the spore forming thermophilic bacteria belong to *Bacillus* and *Clostridium* genera being the first ones, many strains have been characterized [4,5].

Most of the studied thermophilic bacteria have been classified in Group 5 belongs to *Bacillus* genus. Thermophilic bacteria of this genus are aerobic or facultative anaerobic, gram positive and in spore forming form, and have been classified in Group 1 and 5. As a result of phenotypic and phylogenetic studies performed on the basis of thermophilic microorganisms of Group 5, *Bacillus* genus was reclassified according to the 16S rDNA sequence analyses data of *Bacillus* genus as *Amphibacillus* [6], *Alicyclobacillus* [7], *Paenibacillus* [8], *Aneurinibacillus* and *Brevibacillus* [9], *Halobacillus* [10], *Virgibacillus* [11], *Gracilibacillus* and *Salibacillus* [12], *Anoxybacillus* [13], *Coprobacillus* [14], Thermobacillus [15], *Filobacillus* [16], *Geobacillus* [17], *Ureibacillus* [18], *Jeotgalibacillus* and *Marinibacillus* [19,20].

Especially, fast developments in Molecular Biology have caused the usage of DNA based technologies like Ribotyping, ARDRA, quantitative DNA hybridization, rep-PCR, 16S rDNA analyses in the resaerches of microbial diversity in addition to classic morphological, biochemical and physiological methods. And, this has provided possibility for microorganisms at species and subspecies levels to be discriminated more reliably [2, 21, 22]. Among these, rep PCR [REP (repetitive extragenic palindromic), ERIC (enterobacterial repetitive intergenic consensus) and BOX elements] method is a method which is performed to present the most of the Actinomycetes, gram positive and negative bacteria's genomic differences. This method is a very and effective method for studying the diversity in ecosystem, presenting the phylogenetic relation between strains and discriminating the microorganism which are genetically close to each other [2, 23].

The objective of the present study was to isolate, identify and characterize thermophilic bacterial strains present in Hasanabdal hot spring water, Van, Turkey by using molecular methods.

Material and Methods

Isolation of Strains

The water and sludge samples used in this study were inoculated into Nutrient Broth (NB) by bringing them to the laboratory under aseptic conditions, and incubated in aerobic and anaerobic incubators with an adjusted temperature of 55-60°C for 24-48 hours. Then, by culturing 3-4 phases into Nutrient Agar (NA) containing petri dishes, colonies that were thought to be different than each other were purified. Their colony and cell morphologies were examined under microscope, and the colonies which are different than each other were stored in glycerol containing NB at -86°C until being used.

Physiological and Biochemical Characterizations of Isolates

The temprature range for growth was determined by incubating the isolate from 30 to 85°C. The effect of NaCl on the thermophilic bacterial growths were studied in NB medium containing 2.0, 3.0, 4.0, 5.0, 8.0, 10.0 % (w/v) NaCl. The pH dependence of growth was tested in the pH range 4.0-11.0 in nutrient broth medium. Gram reactions, the presence of

catalase, oxidase and amilase reduction were investigated according to the methods described by Fahy and Hayward [24].

Extraction and analysis of FAMEs

Preparation and analysis of FAME from whole cell fatty acids of bacterial strains were performed according to the method described by the manufacturer's manual (Sherlock Microbial Identification System version 4.0, MIDI, Inc., Newark, DE, USA) [25, 26]. FAMEs were separated by gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA, USA) with a fused-silica capillary column (25 m x 0.2 mm) with cross-linked 5% phenyl methyl silicone. FAME profiles of each bacterial strain were identified by comparing the commercial databases (TSBA 40) with the MIS software package. The identity of bacterial strains was revealed by computer comparison of FAME profiles of the unknown test strains with those in the library.

DNA Extraction from Pure Cultures

Total genomic DNA was extracted from bacteria samples using a modified method described by Khoodoo *et al*. [2, 27].

rep-PCR (REP and BOX-PCR) Analysis

A total of 10 isolates were subjected to rep-PCR genomic fingerprinting using primer sets corresponding to REP and BOX elements [28]. The 18-mer primer pair REP 1R (5'-IIIICGICGICATCIGGC-3') and REP 2 (5'-ICGICTTATCIGGCCTAC-3') (where I is Inosine) and BOX1A (5'-CTACGGCAAGGCGACGCTGA CG-3') were used to amplify putative REP-, ERIC- and BOX-like elements in bacterial DNA, respectively. Briefly, approximately 50 ng of purified DNA was used as a template in a 30 µl reaction mixture. Twenty seven µl of reaction cocktail was prepared as follows: Gitschier Buffer 5 µl, Dimethyl sulphoxide 2.5 µl (100%), dNTPs (10mM) 1.25 µl, Bovine serum albumin 1.25 µl (20 mg/ml), primer/primers (5µM) 3.0 µl, taq polymerase (250unit) 0.3 µl, water 10.7 µl (for BOX PCR, 13.7 µl). PCR amplification reactions were performed with a Corbett Research Palm Cycler (Corbett CG1-96 AG, Australia) using the following conditions: an initial denaturation at 95°C for 7 min; 30 cycles consisting of 94°C for 1 min and annealing at 40 or 53°C for 1 min with either REP or BOX primers, respectively; extension at 65°C for 8 min; and a sing1e final polymerization at 65°C for 15 min before cooling at 4°C.
To ensure consistency in results, PCR was repeated for each isolate for at least three times

Electrophoresis

The PCR products (10 µl) were mixed with 6xgel loading buffer (3 µl) and loaded onto an agarose (1.5% w/v) gel electrophoresis in 0.5XTAE (Tris-Acetat- EDTA) buffer at 40 V for 200 min. Amplification products separated by gel was stained in ethidium bromide solution (2 µl EtBr/100ml 1XTAE buffer) for 40 min. The amplified DNA product was detected by using the DNR-Imaging System with UV-soft analysis package (Israel).

Data Analysis of rep-PCR Result

PCR products were scored as presence (1) and absence (0) of band for each of the 6 accessions analyzed. Data were used to calculate a Jaccard (1908) similarity.

All of the experiments in this study were repeated at least twice.

PCR Amplification and Cloning of 16S rDNA Sequence

The 16S rDNA genes were selectively amplified from purified genomic DNA by using oligonucleotide primers designed to anneal to conserved positions in the 3' and 5' regions of bacterial 16S rDNA genes. The forward primer, UNI16S-L (5'-ATTCTAGAGTTTGATCATGGCTCA), corresponded to positions 11 to 26 of *Escherichia coli* 16S rDNA, and the reverse primer, UNI16S-R (5'-ATGGTACCGTGTGACGGGCGGTGTGTA), corresponded to the complement of positions 1411 to 1393 of Escherichia coli 16S rDNA [29]. PCR reaction conditions were carried out according to Beffa *et al*. [30] and the PCR product was cloned to a pGEM-T vector system.

Sequencing and Phylogenetic Analysis

Following PCR amplification and cloning of the 16S rDNA genes of our isolates, the 16S rDNA gene sequences were determined with an Applied Biosystems model 373A DNA sequencer by using the ABI PRISM cycle sequencing kit (Macrogen, Korea). The sequences consisting of about 1397-1414 nucleotides (nt) of the 16S rDNA gene was determined. These sequences were compared with those contained within GenBank [31] by using a BLAST search [32].

Result and Discussion

From the purified isolates, the microorganisms which showed differences in terms of colony and cell morphologies were subjected to various physiological and biochemical tests. As a result, it was determined that all of them (P56, P60, P59, P22, P86, P4, P13, P35, P14 and P9) are gram, catalase, oxidase positive and endospore forming, mobile rods, and P13 isolate is amylase positive, but the rest of the microorganisms are amylase negative. As a result of pH and salt tests, it was determined that for all of the isolates, the optimum pH is 7,5-8,5, and all of the bacteria propagate well in the range of salt concentration of 2-5%. At the end of temperature tests, it was determined that for all of the isolates, the optimum temperature is 56°C. These met the criteria of thermophilic bacteria, which grow at temperatures above 50°C [33].

To verify the systematic position of this bacterium, a study of morphological and physiological characteristics, cellular fatty acids analysis (FAMEs), rep-PCR (REP and BOX-PCR), and 16S rRNA sequence analysis were undertaken.

Totally, 15 different FAMEs were detected in 10 bacterial strains, which were tested in the present study (Table 1). As a result of the FAMEs analyses of the microorganisms, it was determined that for P13 and P9 isolates, iso-C15: 0, anteiso-C15: 0, iso-C17:0, and anteiso-C17: 0 saturated fatty acids (13.34-27.55%) are the major fatty acids; they constitute the 73.79% and 75.89% of the total fatty acid content respectively. These strains were identified as *Bacillus* spp. On the other hand, for P22, P86, P56, P60, P59 and P4 isolates, C15:0, iso-C16:0, and iso-C17:0 fatty acids (20.13-29.80%) are the major fatty acids; they constitute the 65.97-76.51% of the total fatty acid content. These strains were identified as members of the *Geobacillus*. And, for P14 and P35 isolates, iso- C15:0 and anteiso-C15: 0 fatty acids (25.18-31.27%) are the dominating ones; they constitute the 77.33% and 73.42 % of the total fatty acid content respectively. These strains were identified as members of the *Brevibacillus*. These results showed that FAMEs analysis is an appropriate phenotypic method for the discrimination of *Bacillus*, *Geobacillus* and *Brevibacillus* spp. strains at genus level but not at species level.

Fatty acid concentration (%)										
Fatty acids	**P22**	**P86**	**P4**	**P13**	**P56**	**P59**	**P60**	**P14**	**P9**	**P35**
14:0	2.65	-	1.21	4.46	-	2.64	3.08	-	4.96	3.12
14:0 iso	-	1.45	0.99	-	-	-	-	2.11	-	-
15:0 iso	8.12	7.85	7.58	27.55	6.12	7.58	8.99	25.18	26.21	42.15
15:0 anteiso	7.56	6.03	8.87	21.11	7.69	6.23	7.21	52.15	20.13	31.27
15:0	24.25	23.21	26.23	-	29.8	25.08	26.58	-	-	1.32
16:0 iso	20.13	21.69	23.28	8.05	24.5	22.12	23.61	2.83	7.03	3.05
16:0	2.45	3.02	2.11	7.36	2.66	-	-	1.99	5.88	1.23
17:0 iso	21.59	22.53	22.09	12.25	22.21	21.65	22.88	2.15	14.11	2.08
17:0 anteiso	8.64	7.56	1.36	14.98	-	5.89	-	5.27	13.34	6.12
16:1 w7c	-	-	1.28	-	1.55	2.36	1.67	3.35	2.16	3.09
16:1 w11c	1.01	1.98	1.51	1.09	1.48	2.89	2.39	2.02	3.52	2.11
17:1 w10c	1.38	2.87	2.08	0.99	2.05	2.01	1.48	3.11	1.24	2.34
17:1 iso 1/Antei B	1.31	1.12	1.42	1.23	1.17	1.09	0.97	-	2.06	2.02
17:1 iso w10c	-		-	-	-	-	-	-	-	-
19:0 iso	1.01	0.69	-	-	0.91	0.85	1.09	-	-	-

Table 1. Cellular fatty acid composition (%w/w) of strains

As we compared the obtained results of FAME with the literature data [2, 17, 34-40], it was concluded that P13 and P9 isolates are strains that may belong to *Bacillus* genus, P14 and P35 isolates are strains that may belong to *Brevibacillus* genus, and P22, P86, P56, P60, P59 and P4 isolates may belong to *Geobacillus* genus.

Repetitive element PCR (rep-PCR) profiles based on BOX regions have been used for typing, systematic, and epidemiology studies [41-43]. In the direction of this information, REP and BOX PCR were performed to determine the genomic differences between the strains (Figures 1-2). REP–primer pair generated 1–7 fragments of size 200–4200 bp and faint bands were frequently observed (Figure 1). Primer BOX-A1R generated 5–11 fragments of size 250–4500 bp (Figure 2).

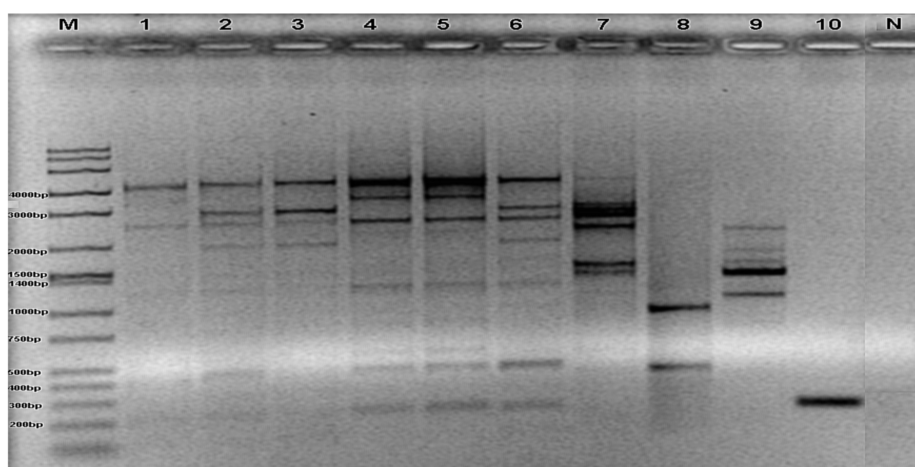

Figure 1. REP-PCR profile generated with the REP 1R and REP 2 primers. Lanes: 1) P56; 2) P60; 3) P59; 4) P22; 5) P86; 6) P4; 7) P13; 8) P35; 9) P14; 10) P9; N; Negative Control; M) Molecular Marker (10kb)

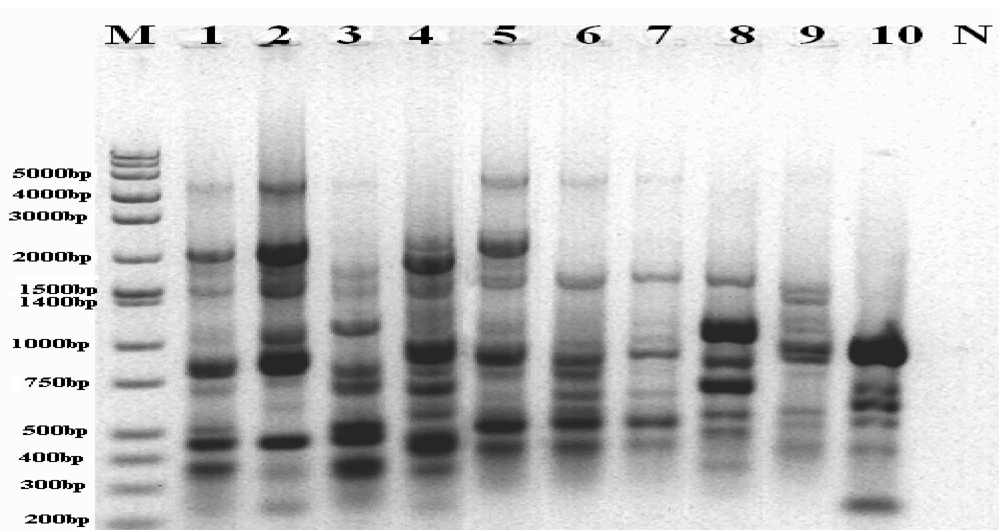

Figure 2. BOX-PCR profile generated with the BOX A1R primer. Lanes: 1) P22; 2) P86; 3) P4; 4) P13; 5) P56; 6) P59; 7) P60; 8) P14;9) P9; 10) P35; N; Negative Control; M) Molecular Marker (10kb)

In addition to the random amplified polymorphic DNA (RAPD-PCR) technique, rep-PCR genomic fingerprint protocols were performed for the closely related *Bacillus*, *Brevibacillus* and *Geobacillus* genera exhibiting high sensitivity in the discrimination of mesophilic and thermophillic species at the strain level [43-46].

In their study, Von der Weid *et al*. [47] tried to show the genetic difference between the isolates belong to *Paenibacillus polymyxa* species by BOX PCR method and observed that this method clearly indicates the discrimination between the strains.

In their study, Cherif *et al*. [48] determined that rep-PCR analysis is a useful tool to describe the genetic diversity and the genetic relationship of closely related bacteria such as the members of the *Bacillus cereus* group. In this study, the difference between the strains of *Geobacillus*, *Brevibacillus* and *Bacillus* genera was clearly determined similar to the literature data by rep PCR method.

In the literature, Lopez and Alippi [49] tried to show the genetic differecences between the isolates belong to *Bacillus cereus* species by rep-PCR method, and it was observed that this method clearly indicates the discrimination between the strains.

With this study, in contrary to Meintanis *et al*. [43] who determined that the REP-PCR method generated more informative results than BOX-PCR for the studied strains, and REP-PCR profiles were more distinct for the different strains, we found that for the studied strains, the BOX-PCR method provided more informative results than REP-PCR; the BOX-PCR profiles of the different strains were more distinct, including a higher number of bands.

At the end of cluster analysis that was performed by taking into account the data of BOX-PCR by which the highest number of polymorphic bands were observed, it was determined that the tested bacteria were classified in 4 clusters (Figure 3). It was determined that in the first cluster, there are totally four strains (P59, P60, P56, P22 and P4), and the ratio of similarity between them is 79-99%. And, there are two strains (P14-P9) in the second cluster, one strain (P13) in the third cluster, and one strain (P35) in the fourth cluster in total.

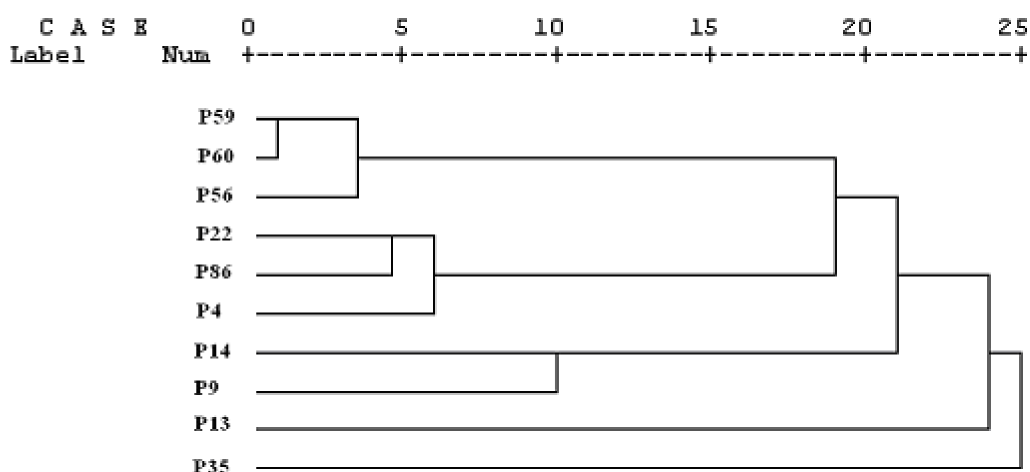

Figure 3. BOX-PCR Cluster Analyses

By examining the rep-PCR data, it was detected that P59, P60, P56, P22, P86 and P4 strains, which were previously detected by FAMEs that they belong to *Geobacillus* genus, belong to same species. From these strains, the ones that are least similar to each other according to cluster analysis data (P22, P86 and P4) and the ones that are in different clusters (P14, P9, P13 and P35) were subjected to sequence analysis.

As a result of this study, it was detected that from the 10 isolates, isolated from Van city Ercis town Hasanabdal hot springs, P22, P86, P56, P60, P59, and P4 strains belong to *G. pallidus* species, P13 belongs to *Bacillus licheniformis*, P9 belongs to *B . pumilus*, P14 belongs to *Brevibacillus brevis*, and P35 belongs to *Br. borstelensis* species.

Regarding the 16S rRNA gene sequences of *Geobacillus*, *Bacillus* and *Brevibacillus* test isolates, the similarity ratio of 99% was retrieved (Table 2) confirming Zeigler [50] and Meintanis *et al*. [43] who confirmed that the 16S rRNA gene sequences similarity of *Geobacillus* and *Bacillus* type strains is higher than 98.5%. Although the 16S rRNA gene is used as framework for modern bacterial classification, it has often been seen that its usage shows limited variation for the discrimination of closely related taxa and strains [43, 51].

Table 2. The comparison of the 16S rDNA gene sqeuences of the obtained isolates with the 16S rDNA gene sequences in the gene bank

Sequence				
Code Numbers of Test Microorganisms	**GenBank accession number**	**Number of Nucleotides***	**Percent identity****	**Closest phylogenetic relative (GeneBank accession number)**
P22	FJ417405	1409	99%	*Geobacillus pallidus*
P86	FJ417407	1413	99%	*Geobacillus pallidus*
P4	FJ417401	1413	99%	*Geobacillus pallidus*
P13	FJ417403	1412	99%	*Bacillus licheniformis*
P14	FJ417404	1398	99%	*Brevibacillus brevis*
P9	FJ417402	1410	99%	*Bacillus pumilus*
P35	FJ417406	1398	99%	*Brevibacillus borstelensis*

*The number of 16S rDNA nucleotides used for the alignment.
**The percentage identity with the 16S rDNA sequence of the closest phylogenetic relative.

As the phenotypic and genotypic analyses data were examined, it was seen that the results of FAMEs were inefficient in terms of identification, but by considering the major fatty acids,

they gave a brief idea about the genera to which the test isolates may belong. Then, it was determined that the BOX-PCR method more successfully exhibits the discrimination between the isolates at species and subspecies levels than REP-PCR method.

However, this is the first study demostrated that *B. licheniformis* and *G. pallidus*, *B. pumilus*, *Brevibacillus brevis* and *Br. borstelensis* populations are five common bacterial species present in Hasanabdal hot spring water.

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