THE GENETIC DIVERSITY OF GENUS BACILLUS AND THE RELATED GENERA REVEALED BY 16S rRNA GENE SEQUENCES AND ARDRA ANALYSES ISOLATED FROM GEOTHERMAL REGIONS OF TURKEY

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ABSTRACT

Previously isolated 115 endospore-forming bacilli were basically grouped according to their temperature requirements for growth: the thermophiles (74%), the facultative thermophiles (14%) and the mesophiles (12%). These isolates were taken into 16S rRNA gene sequence analyses, and they were clustered among the 7 genera: Anoxybacillus, Aeribacillus, Bacillus, Brevibacillus, Geobacillus, Paenibacillus, and Thermoactinomycetes. Of these bacilli, only the thirty two isolates belonging to genera Bacillus (16), Brevibacillus (13), Paenibacillus (1) and Thermoactinomycetes (2) were selected and presented in this paper. The comparative sequence analyses revealed that the similarity values were ranged as 91.4-100 %, 91.8- 99.2 %, 92.6- 99.8 % and 90.7 - 99.8 % between the isolates and the related type strains from these four genera, respectively. Twenty nine of them were found to be related with the validly published type strains. The most abundant species was B. thermoruber with 9 isolates followed by B. pumilus (6), B. licheniformis (3), B. subtilis (3), B. agri (3), B. smithii (2), T. vulgaris (2) and finally P. barengoltzii (1). In addition, isolates of A391a, B51a and D295 were proposed as novel species as their 16S rRNA gene sequences displayed similarities ≥ 97% to their closely related type strains. The AluI-, HaeIII- and TaqI- ARDRA results were in congruence with the 16S rRNA gene sequence analyses. The ARDRA results allowed us to differentiate these isolates, and their discriminative restriction fragments were able to be determined. Some of their phenotypic characters and their amylase, chitinase and protease production were also studied and biotechnologically valuable enzyme producing isolates were introduced in order to use in further studies.

Key words: isolation, temperature requirement, endospore-forming bacilli, 16S rRNA gene, ARDRA

INTRODUCTION

The genus Bacillus is a phenotypically large, diverse collection of Gram-positive or Gram-variable staining, endospore-forming, aerobic or facultatively anaerobic, rod-shaped bacteria that have undergone considerable recategorization, and the advances in molecular biology have revealed a high phylogenetic heterogeneity (5, 21). The genus Bacillus *Corresponding Author. Mailing address: Ankara University, Faculty of Science, Department of Biology, 06100, Tandogan, Ankara, Turkey.; Tel: +90 312 2126720/1095; Fax: +90 312 2232395.; E-mail: arzucoleri@gmail.com / acihan@science.ankara.edu.tr
and related genera are distributed widely in nature and include thermophilic, psychrophilic, acidophilic, alkalophilic and halophilic bacteria that utilize a wide range of carbon sources for heterotrophic growth or grow autotrophically.

The investigations on phylogenetic divergence of the genus *Bacillus* and its mesophilic and thermophilic members indicated the need for further and extensive studies to place some of these bacilli in appropriate taxonomic levels (1, 23, 21). With the accumulation of further 16S rRNA gene sequence data, *Bacillus* has been divided into more manageable and better-defined groups (16). According to Ludwig et al. (2007) and Logan et al. (2009), the phylum Firmucutes, class “Bacilli”, order *Bacillales*, included seven named families that may lie within the taxonomy of the genus *Bacillus* and related organisms (16, 17). With the newly named families containing the genera of the aerobic and endospore forming bacteria were finally reclassified as: i) *Bacillaceae* (6), ii) *Alicyclobacillaceae* (36), iii) *Paenibacillaceae* (2), iv) *Planococcaceae* (13), v) *Thermoactinomycetaceae* (32), vi) *Pasteuriaceae* (18) and viii) *Sporolactobacillaceae* (11).

In addition, using 16S rRNA gene sequence analysis, Ash et al. (1991) described the presence of five phylogenetically distinct groups in the genus *Bacillus*, and Nielsen et al. (1994) subsequently described a sixth group belonging to the alkaliphilic bacilli (1,2). Molecular analysis showed that the majority of mesophilic bacteria described in the literature belonged to the genus *Bacillus* genetic groups 1 and 3 (1). The facultatively thermophilic species *Bacillus smithii*, *Bacillus coagulans* and *Bacillus licheniformis* fall into group 1 along with other mesophilic species such as *Bacillus subtilis* (23). In addition, ribosomal DNA genetic group 3 was comprised from mesophilic species of genus *Paenibacillus* (1). In 2001, the thermophilic bacteria belonging to *Bacillus* genetic group 5 were reclassified as being members of the genus *Geobacillus* (21). Since then, the thermophilic members having growth optimum in the temperature range from 45 to > 70 °C are classified into the genera *Bacillus*, *Aeribacillus*, *Anoxybacillus*, *Cerasibacillus*, *Caldalkalibacillus*, *Alicyclobacillus*, *Sulfobacillus*, *Brevibacillus*, *Ureibacillus*, *Thermobacillus* and *Thermoactinomyces* (16, 21, 19).

Furthermore, *Bacillus* and related genera are one of the mostly interested groups of bacteria in industrial biotechnology on behalf of their enzymes most of which showed resistance to high pH and temperature values especially in harsh industrial processes (27). Therefore, polyphasic approach has been taking attention in microbial ecological researches in order to screen isolates producing novel enzymes that could promise to use in new applications (9). *Bacillus* and related genera are able to produce endospores how resistance forms from unfavorable life conditions; they can survive and be isolated from extreme environments such as hot, cold, acidic, alkali and saline areas. Consequently, it is not surprising to isolate mesophilic members of endopore-forming bacilli from geothermal habitats along with their thermophilic counterparts.

During a polyphasic study, more than five hundred thermophilic and mesophilic endospore-forming bacilli were isolated from different geothermal regions of Turkey (7). In the present study, one hundred and fifteen of them were taken into the 16S rRNA gene sequence analyses. Among these isolates, mostly the mesophilic and facultative thermophilic thirty two bacilli belonging to genera *Bacillus*, *Brevibacillus*, *Paenibacillus* and *Thermoactinomyces* were then selected and taken into further analyses. The taxonomic data of these endospore-forming bacilli presented in this paper were derived from the phenotypic characteristics, 16S rRNA gene sequences and amplified ribosomal rDNA restriction analyses (ARDRA). Isolates were also screened for their amylolytic, glucosidic, proteolytic and chitinolytic activities which might have biotechnological potential.

**MATERIALS AND METHODS**

**Sampling, isolation and growth conditions**

The sampling, isolation and growth conditions of the
isolates used in this study were as follows: A total of 32 samples including water (4), sediment (11), soil (16) and stone samples in diameters from 2 to 3 cm within the hot spring (1) were collected aseptically from 11 hot springs and 9 high-temperature well pipelines located in two geographically separated areas in Turkey: Aegean Region and Middle Anatolian Region. Among these geothermal regions, Aydın (Region A; 27º 51´ E, 37º 51´ N), Manisa (Region B; 27º 26´ E, 38º 36´ N), Denizli (Region C; 29º 06´ E, 37º 46´ N) and İzmir (Region D; 27º 09´ E, 38º 25´ N) provinces are in the Aegean Region, whereas Nevşehir (Region E; 34º 43´ E, 38º 38´ N) and Ankara (Region F; 32º 52´ E, 39º 56´ N) provinces are located in the Middle Anatolian Region of Turkey. The water temperature and pH of these geothermal areas were measured between 50-100 ºC and 6.0-9.0, respectively.

One ml water and sediment, 1 g soil or stone samples were incubated in 5 ml of the MI medium containing 1 % soluble starch, 0.5 % peptone, 0.3 % yeast extract, 0.3 % meat extract, 0.3 % K₂HPO₄ and 0.1 % KH₂PO₄, (pH 7.0) at 37 ºC, 45 ºC and 60 ºC under shaking for 24 h to obtain the enrichment culture, after each sample was heat-treated at 80 ºC for 10 min to kill vegetative cells (7, 30). The turbid enrichments were streaked on plates of MI medium containing 3 % agar and incubated aerobically at 37 ºC, 45 ºC and 60 ºC for 24-48 h. The single colonies showing different colony morphology were isolated and subcultured at least three times until a pure culture was obtained. The cultures of the isolates were monitored by microscopy analysis. All of the isolates were routinely maintained at 4 ºC on MI agar slants and stored at -80 ºC in MI broth cultures supplemented with 20 % glycerol. Isolates were designated according to their geothermal area of origin, the sample number taken from that origin and the number of the isolates obtained in that sample. The designation of the 34 isolates, their origin, and the reference strains used in this study are presented in Table 1.

### Table 1. Diversity and origin of the bacilli isolated from various geothermal regions of Turkey and the reference strains used in this study

| Bacterial isolates | Origin | Number of the isolates and strains |
|--------------------|--------|----------------------------------|
| A111α, A181α, A296α | Omerbeyli, Germencik, Aydın, Turkey | 3 |
| A381α, A391α | Yavuzkoy, Salavatlı, Aydın, Turkey | 2 |
| B51αβ, B66α, B91α, B93α | Urganlı, Turgutlu, Manisa, Turkey | 4 |
| C83ca, C292b | Buharkent, Tekkehamam/Tekkeköy, Denizli, Turkey | 2 |
| D75α, D75b | Balçova Geothermal Site, İzmir, Turkey | 2 |
| D45α | Seferhisar, Urkmez, İzmir, Turkey | 1 |
| D273α, D295b, D311α, D662b | Seferhisar, Doganbey, İzmir, Turkey | 4 |
| D194α, D194b, D505α, D505b | Dikili, Kaynarca, Kocaoba, İzmir, Turkey | 4 |
| D362b | Dikili, Zeytindalı, İzmir, Turkey | 1 |
| E114β, E287b, E302α, E308α, E3010cα | Altınsu, Kozaklı, Nevşehir, Turkey | 5 |
| E165α, E187α, E215α | Baglıca Kozaklı, Nevşehir, Turkey | 3 |
| F92α | Kızılcahamam, Ankara, Turkey | 1 |
| Total number of the bacterial isolates | | 32 |

Reference strains

- *Bacillus amyloliquefaciens* DSM7™
- *Bacillus licheniformis* DSM 13™
- *Bacillus coagulans* DSM 1™
- *B. subtilis* DSM 347™

α; soil sample, β; sediment sample, γ; water sample, δ; stone samples in the hot spring
Morphologic and physiologic characterization

The temperature range was determined by incubating the strains in Nutrient Agar at temperatures from 20 to 80 °C for 24-72 h. All the incubation conditions were adjusted to 37 °C, 45 °C or 60 °C according to the temperature requirements of the mesophilic, facultative thermophilic and the thermophilic isolates, respectively. The cell morphology, motility and spore formation were observed with freshly prepared wet mounts using phase-contrast microscopy. The active cultures grown in Nutrient Broth under shaking for 18-24 h were used when describing the cell morphology and motility after incubation. The formation of spores was also tested by using Nutrient Broth cultures of 18-48 h supplemented with 5 mg/l MnSO$_4$·4H$_2$O (5). The colony morphologies were determined using cultures grown aerobically on Nutrient Agar for 18-24 h. Gram staining and catalase activity were carried out by the methods of Claus and Berkeley as described previously (5). The reference strains were used as control groups in all the phenotypic and genotypic characterization tests which were at least triplicate.

Enzyme assays

All the isolates were screened for their chitinase, amylase, α-glucosidase and protease activities qualitatively on agar plates at incubation conditions adjusted to the temperature requirements of the isolates. 1.6 % (w / v) Nutrient Broth containing 0.2 % (w / v) colloidal chitin (24) was used for chitinase production. Chitinase production was detected by observing clear zones around the colonies after growth for 48-72 h. Amylolytic activity was tested on MI agar plates after incubation for 48 h. Then the plates were treated with 0.2 % I$_2$ in 2 % KI solution and isolates having starch digestion zones around their colonies were determined as amyloytic (7). When determining α-glucosidase activity, a screening was carried out on MI plates by searching para-nitrophenol α-D-glucopyranoside (pNPG) activity on blotting filter paper as described previously (4). The paper disk was incubated at 40 °C or 60 °C and the yellow color formation, the color of which was caused by the reaction of α-glucosidase on the substrate, was observed and selected for the positive α-glucosidase reaction. In the screening of protease activity, isolates were growth on Skim Milk Agar (pH 7.0) plates for 72 h (8). Protease producing isolates which gave a clear zone around their colonies due to the hydrolysis of skim milk were selected. The diameters of halo zones and the amount of yellow color formation were also measured and compared with the reference strains producing these enzymes.

16S rRNA gene amplification and sequencing analyses

Genomic DNA was extracted from the cultures growing on Nutrient Agar for 18 h according to the temperature requirement of the isolates by using genomic DNA purification kit (Fermentas). The gene encoding 16S rRNA was amplified by PCR with the 16S bacteria specific 27F forward and the 1492R reverse primer as described previously (14). The amplification products were purified from agarose gel using Gel Extraction Kit (Omega Ezna). The sequences of the PCR-amplified 16S rRNA gene were determined by using ABI 3100 gene sequencer with Bigdye cycle sequencing kit. In the phylogenetic analysis, homology search was carried out using the basic BLASTN search program at the NCBI Web-site. Phylogenetic analysis were performed using the maximum-likelihood and neighbor-joining methods with bootstrap values based on 1000 replications and the phylogenetic tree (26) was constructed with the MEGA package version 4 (31) according to Jukes-Cantor method (10).

The GenBank accession numbers

The 16S rRNA gene sequences and their GenBank accession numbers are presented below: A111 (FJ429567), A381a (FJ429999), A391a (FJ430001), B51a (FJ429572), C83ca (FJ429573), D75a (FJ430020), D194a (FJ430022), D311 (FJ430035), D362 (FJ429579), E114 (FJ430054), E215 (FJ429588), E287 (FJ430065), E308 (FJ430066), F92 (FJ430068), D273a (FJ430033), A181 (FJ429991), A296 (FJ429992), B66 (FJ430008), B91 (FJ430010), B93
Amplified ribosomal DNA Restriction Analysis (ARDRA)

ARDRA analysis of the 16S rRNA gene primed by 27F/1492R was carried out on the amplified PCR products by single enzyme digestion, according to the manufacturer’s instructions, with Fast digest AluI, HaeIII and TaqI restriction enzymes (MBI Fermentas). The ARDRA profiles of the digested DNA were analyzed by electrophoresis through 2 % (w/v) agarose gel using 1 X TBE buffer at 120 V/cm for 1.5 h (3). The ARDRA patterns were analyzed by the Bionumerics version 6.1 software packages (Applied Maths, Belgium). The experimental restriction fragments higher than 75 bp were included in the statistical analysis, in order to avoid confusion with primer dimer bands. Similarities of the digitized profiles were calculated using Dice correlation and an average linkage (UPGMA) dendrogram was obtained. All the restriction analyses and their agarose gel electrophoresis were carried out in triplicates. In addition to experimental restriction analyses, the theoretical restriction mapping of the analyzed 16S rRNA gene sequences were also carried out by using an online restriction mapping service (http://restrictionmapper.org/).

RESULTS

Temperature requirements of isolated strains

One hundred and fifteen isolates were basically classified into three groups according to their temperature requirements; the mesophiles (20 - 45 °C, T_{opt}; 30 - 40 °C), the facultative thermophiles (25 - 60 °C, T_{opt}; 40 - 50 °C) and the thermophiles (40 - >70 °C, T_{opt}; 50 - >70 °C). Among the geothermal areas studied, majority of the isolated bacilli (85 of 115 isolates) showed thermophilic growth with optimum temperature values from 50 to >70 °C. The number of the isolates being facultative thermophilic and mesophilic were relatively lower than the thermophiles. Sixteen of these isolates were found to be facultative thermophilic (A111, B51a, C83ca, E114, D362, F92, A181, A296, B66, B91, B93, C292, D45, D662b, E165, D295), whereas 14 of them showed mesophilic growth (D311, E287, E215, A381a, D75a, D75b, D194a, D194b, E308, A391a, D505a, D505b, E187 and D273a).

Selection of isolates from genus Bacillus and other Bacillus-related genera according to the 16S rRNA gene sequence analyses

In order to determine their phylogenetic position, the 16S gene sequence was analyzed for all 115 endospore-forming isolates. All of them were phylogenetically clustered on the basis of their individual 16S rRNA gene sequence homologies to their closest relatives. According to the phylogenetic analysis of these sequences, most of the isolates fell into Bacillus genetic group 5 along with other thermophilic species. The other isolates clustered in Bacillus genetic group 1 and 3 with their mesophilic and facultative thermophilic counterparts. Comparison of the generated sequences with those in the GenBank database indicated that all of the identified isolates from geothermal regions of Turkey were belonged to the families Bacillaceae, Paenibacillaceae and Thermoactinomycetaceae from order Bacillales. One hundred and twelve of them were clustered among the 7 genera and grouped according to their temperature requirements for growth: the thermophiles from genera Anoxybacillus (52 isolates), Geobacillus (27), Aeribacillus (4) and Thermoactinomycetes (2); the facultative thermophiles from genera Brevibacillus (10) and Bacillus (6); and finally the mesophiles from genera Bacillus (10), Brevibacillus (3) and Paenibacillus (1).

Among these isolates, thirty two mostly mesophilic or facultative thermophilic, endospore-forming isolates from genera Bacillus, Brevibacillus, Thermoactinomycetes and Paenibacillus were selected and taken into further researches for this study. These selected isolates, which were presented in this study, were totally obtained from 20 geothermal sampling stations: 11 hot springs and 9 high temperature well pipelines,
located in the Aegean Region and Middle Anatolian Region in Turkey. Mostly completely sequenced (1362-1404 bp) 16S rRNA gene sequence data of these isolates have been deposited in the GenBank databases and their accession numbers in relation to the isolates were given in the phylogenetic tree (Fig. 1).

![Phylogenetic tree](image)

**Figure 1.** A phylogenetic tree based on the 16S rRNA gene sequences between isolates belonging to genus *Brevibacillus*, *Paenibacillus*, *Thermoactinomycetes*, *Bacillus* and the related members from these genera. The tree was generated by neighbour-joining method. Bootstrap values (%) are based on 1000 replicates and shown for branches with more than 45 % bootstrap support. Bar indicates 0.01 substitutions per 100 nucleotide positions.
The genetic diversity of the isolates belonging to genera *Bacillus*, *Brevibacillus*, *Thermoactinomyces* and *Paenibacillus*

As the isolated bacteria were originated from hot environments, representatives of the genera *Bacillus*, *Brevibacillus*, *Thermoactinomyces* and *Paenibacillus* were significantly less-correspondingly 16, 13, 2 and 1, when compared with the thermophilic members among the geothermal regions in Turkey (Fig. 1). The phylogenetic analyses derived from neighbor-joining method were congruent with those obtained using the maximum-likelihood algorithms. Thus, the phylogenetic tree only constructed with the neighbor-joining method is presented in this study. The divergence of the species in these genera, their 16S rRNA gene sequence similarity values to their closest relatives and the number of the isolates belonging to the species groups are all given in Table 2. Comparative sequence analyses revealed that the sequence similarity values between isolates and type strains from genus *Bacillus* were 91.4 % to 100 %. *Bacillus* isolates also demonstrated 16S rRNA gene sequence similarities from 85.8 % to 99.9 % to each other. From genus *Bacillus* totally 4 species groups were observed: 7 of the isolates were found to be related to *Bacillus pumilus*, 4 to *Bacillus licheniformis*, 3 to *Bacillus subtilis* and 2 to *Bacillus smithii* with sequence similarity values presented in Table 2. Only six of the isolates were within the facultatively thermophilic species of *B. licheniformis* and *B. smithii* which fall into *Bacillus* genetic group 1 with other mesophilic strains like *B. subtilis*. Isolate A391a from *B. pumilus* group and B51a from *B. licheniformis* grouped as separate clusters in the phylogenetic tree and represented two novel species as concluded from their low sequence similarity values to their closest relatives such as 96.7 % and 95.3 %.

Isolates from members of the genus *Brevibacillus* were diverged into two groups with 16S rRNA gene sequence similarity values of 91.8 % to 99.2 % to all the described *Brevibacillus* type strains and 91.6 % to 99.9 % to each other. One of which contained the facultatively thermophilic species *Brevibacillus thermoruber* (9 isolates with similarity values of 97.5-99.1 %). The other members of this genus were belonged to mesophilic *Brevibacillus agri* species (3 isolates having similarity values of 98.6-99.2 %).

In addition, 2 of the isolates were found to be belonged to species *Thermoactinomyces vulgaris* and 1 from species *Paenibacillus barengoltzii* with 16S rRNA gene sequence similarity values of 97.3 % - 99.8 % and 99.8 % to their closely related species, respectively.

**Table 2.** The species groups of the genus *Bacillus* and *Bacillus*-related isolates, the intragenic sequence similarity values and the number of the bacteria belonging to these groups derived from 16S rRNA gene nucleotide sequence

| Genus         | 16S rRNA gene grouping       | 16S rRNA gene sequence similarities to the closest relative (%) | Number of the isolates |
|---------------|-------------------------------|---------------------------------------------------------------|------------------------|
| *Bacillus*    | 1- *B. smithii* group         | 94.0-99.6                                                     | 2                      |
|               | 2- *B. pumilus* group         | 96.7-99.7                                                     | 7                      |
|               | 3- *B. subtilis* group        | 94.2-100                                                      | 3                      |
|               | 4- *B. licheniformis* group   | 95.3-99.5                                                     | 4                      |
| *Brevibacillus* | 1- *B. thermoruber* group   | 97.5-99.1                                                     | 10                     |
|               | 2- *B. agri* group           | 98.6-99.2                                                     | 3                      |
| *Thermoactinomyces* | 1- *T. vulgaris* group     | 97.3-99.8                                                     | 2                      |
| *Paenibacillus* | 1- *P. barengoltzii* group | Isolates belonging to genus *Thermoactinomyces* 99.8 | 1                      |
|               |                               | Isolates belonging to genus *Paenibacillus* 99.8 | 1                      |
|               |                               | Total                                                         | 32                     |
Phenotypic characteristics of the isolates

All the isolates from genera Bacillus were observed to be Gram positive, motile and endospore-forming rods. Among the isolates from genus Bacillus, colony morphology of B. pumilis members differed peculiar to the isolate and round colonies, producing cream or yellow pigments, were formed. Subterminally located ellipsoidal endospores were observed from non swollen sporangia. Only spores of the A391a isolate located terminally in swollen sporangia. Starch hydrolysis was negative except for A391a isolate. A391a also differed from other B. pumilis isolates by its ability of producing amylase, α-glucosidase and protease enzymes. Colonies of B. subtilis isolates were in cream color instead of E215 which had white colonies. Ellipsoidal spores of B. subtilis group isolates were subterminally located in non swollen sporangia. Starch hydrolysis was variable. Protease production was a dominant character in B. subtilis group, and E215, D311 and E287 isolates from this group were capable of producing significant levels of proteolytic enzymes. In addition, E287 was also found to be a good amylase producer. Isolates belonging to B. licheniformis group had cream colored colonies except B51a which could produce yellow pigmentation on Nutrient Agar plates. Terminally, subterminally or central located ellipsoidal to oval endospores were observed in swollen or non swollen sporangia. Starch hydrolysis was variable among B. licheniformis isolates. Furthermore, all the B. licheniformis isolates were unexceptionally good protease producers. B. smithii isolates had cream colored, round colonies, produced subterminally located ellipsoidal endospores in swollen or non swollen sporangia, and could not hydrolyze starch.

Isolates of genus Brevibacillus were diverged in colony morphology and spore formation. Starch hydrolysis was weak or negative. Both isolates from B. thermoruber and B. agri produced colonies with cream, pale yellow or yellow color. They also produced subterminally or terminally located oval to ellipsoidal endospores in swollen sporangia.

P. barengoltzii isolate D273a had round cream colored colonies. Terminally or subterminally located oval to ellipsoidal spore formation was observed in swollen sporangia. It was positive for starch hydrolysis. This isolate was unique due to some of its phenotypic characteristics such as high chitinase and amylase production.

E302 and E3010c isolates from Thermoactinomyces genus differed from all the other isolates not only by their colony morphology, but also their cell shape and spore formation. All the isolates used in this study were rod-shaped bacilli except strains E302 and E3010c. Both of these strains produced round spores on the branched mycelium. Endospores were sessile and formed singly. These T. vulgaris isolates had abundant aerial white mycelium. They had a white colored, powdery surface and gradually fading margined colony morphology. Starch hydrolysis was weak in E302 and E3010c isolates.

In addition to these data, except isolates from Thermoactinomyces genus, all the other isolates from Bacillus, Brevibacillus and Paenibacillus showed mesophilic or facultative thermophilic growth. Thermoactinomyces isolates were found to be thermophilic which could grow between 35 °C and 55 °C (T\text{opt} = 50 °C). They also branched with other thermophilic members such as Anoxybacillus and Geobacillus from Bacillus genetic group 5 as can be seen in Fig. 1. Although, all the isolates from B. smithii (25 - 60 °C, T\text{opt} = 55 °C), B. licheniformis (25 - 55 °C, T\text{opt} = 37 °C) and B. thermoruber (35 - 58 °C, T\text{opt} = 46 °C) were facultative thermophilic, they were branched within the Bacillus genetic group 1 with their mesophilic counterparts such as B. subtilis (20 - 40 °C, T\text{opt} = 30 °C), B. pumilis (20 - 45 °C, T\text{opt} = 30 °C) and B. agri (20 - 40 °C, T\text{opt} = 30 °C). Furthermore, Paenibacillus D273a from Bacillus genetic group 3 was also determined to be mesophilic with temperature ranges from 20 - 45 °C and with an optimum value of 37 °C.

AluI-ARDRA profiles of the isolates

Distinctive ARDRA patterns were obtained after restriction with AluI of the amplified 16S rRNA gene of
isolates from the genera *Bacillus*, *Brevibacillus*, *Thermoactinomyces* and *Paenibacillus* and their related cluster analyses, showing the representative profiles, were presented in Fig. 2. According to these experimental-ARDRA results, the four *Bacillus* 16S rRNA gene groups of which the isolates were included formed 4 different *AluI*-ARDRA clusters. Although 8 theoretical-ARDRA groups were observed among the isolates from genus *Bacillus*, four of these excess groups could not be differentiated by experimental analysis. As can be seen in Table 3, the *Bacillus*-AlaI-experimental clustering composition was as follows: cluster 1 included species from *B. smithii* (Ba-A-1, Ba-A-2). The second (237 bp), the third (208 bp) and the fourth (165 bp) restriction bands were all distinctive fragments of this cluster. Isolates from *B. pumilis* branched in cluster 2 which generally shared Ba-A-4 theoretical group in stead of A391a, having the Ba-A-3 theoretical group. The distinctive restriction fragments of this group were the 102 bp (5th) and 87 bp (6th) fragments. Cluster 3 included isolates from *B. subtilis* which could be distinguished from the others by a 98 bp (5th) restriction fragment (Ba-A-5, Ba-A-6). Cluster 4 contained isolates from *B. licheniformis* with a unique 825 bp (1st) restriction fragment which was not observed among the other *Bacillus* species. All the isolates from cluster 4 shared the Ba-A-7 theoretical-ARDRA profile, except E114 (Ba-A-8).

The isolates from genus *Brevibacillus* showed 2 different experimental- and theoretical-ARDRA groups (Fig. 2, Table 3). According to this clustering, all of the *B. thermoruber* isolates in cluster 1 displayed the same experimental restriction pattern and also the same Br-A-1 theoretical profile. The second cluster in genus *Brevibacillus* was formed by the *B. agri* isolates with a similar experimental and theoretical-ARDRA profile (BR-A-2). The distinctive restriction fragments were observed to be 619 bp and 153 bp fragments in *B. thermoruber*, whereas 418 bp, 245 bp and 167 bp fragments differed *B. agri* isolates from *B. thermoruber*. In addition, the isolates from both genera *Paenibacillus* and *Thermoactinomyces* displayed identical theoretical-AlaI-ARDRA profiles with their closely related type species (Table 3).

### Table 3. Some representative theoretical and experimental 16S rRNA gene by *AluI* restriction fragments of isolates belonging to genera *Bacillus*, *Brevibacillus*, *Paenibacillus* and *Thermoactinomyces*

| 16S rRNA gene sequence grouping | Experimental *AluI* | Theoretical *AluI* | Grouping |
|---------------------------------|---------------------|-------------------|----------|
| 1. *B. smithii* group           | 1                   | 2                 | 3        | 4 | 5 | 6 | 7 |
| Genus *Bacillus*                |                     |                   |          |   |   |   |   |
| 1. *B. smithii* group           | D362*               | 454 237 208 165   | 97       | 1 | 316', 213, 162  | Ba-A-1 |
|                                 | F92                 | 450 240 207 166   | 97       | 1 | 428, 208 206 185 177, 140  | Ba-A-2 |
|                                 | TMI12              | nd                |          | 1 | 425, 416 208 185 139  S |
| 2. *B. pumilis* group           | A391a               | 450 290 225 201   | 102 88   | 2 | 428, 382 208 185 87 84  | Ba-A-3 |
|                                 | A381a               | 450 284 226 201 102 87 | 2 | 427, 264 206 185 119 87 84  | Ba-A-4 † |
|                                 | E308                | 450 284 226 201 102 87 | 2 | 427, 264 206 185 122 87 84  | Ba-A-4 † |
| 3. *B. subtilis* group          | E287                | 450 285 225 200 99 | 3 | 425, 264 206 185 172 115  | Ba-A-5 † |
|                                 | E215*               | 450 284 226 204 98 | 3 | 259, 192 180 116  S |
| 4. *B. licheniformis* group     | C83ca               | 825 270 139 115   | 4 | 368, 264 116  S |
|                                 | B51a'               | 825 270 139 115   | 4 | 365, 264 119  S |
|                                 | E114                | 825 270 139 115   | 4 | 825, 264 139 113  S |
|                                 | SK13.02             | 825 270 139 115   | 4 | 429, 264 206 185 172 129  Y |
| Genus *Brevibacillus*           | 1. *B. thermoruber* group | 1 2 3 4 5 6 7   |          |   |   |   |   |
|                                 | C292                | 619 455 212 153   | 1 | 612, 379 206 140  S |
|                                 | D295                | 610 460 210 150   | 1 | 610, 383 206 140  S |
| 2. *B. agri* group             | E187                | 447 418 245 214 167 79 2 | 1 | 394, 381 217 174 160  Y |
| Genus *Paenibacillus*          |                     |                   |          |   |   |   |   |
| P. barengoltzi group            | D273a               | 440 420 215 186 80 | 1 | 417, 384 215 185 87 86  S |
| Genus *Thermoactinomyces*       |                     |                   |          |   |   |   |   |
| T. vulgaris group               | E3010e              | 429 410 402 255 80 | 1 | 414, 403 380 185  T-A-1 † |

(Abbreviations: 1., strains having partial 16S rRNA gene sequences; bold fragment, the distinctive *AluI* restriction fragment; underlined fragment, the 3’ fragment of the 16S rRNA gene sequence; 2., the 5’ fragment of the 16S rRNA gene sequence; Ba-A-#, *Bacillus*-AlaI-# theoretical group; Br-A-#, *Brevibacillus*-AlaI-# theoretical group; P-A-#, *Paenibacillus*-AlaI-# theoretical group; T-A-#, *Thermoactinomyces*-AlaI-# theoretical group; †, the dominant theoretical profile among the distinct 16S rRNA gene groups; nd, not detected. The designation of the novel isolates were showed in bold character)
**HaeIII-ARDRA profiles of the isolates**

The *HaeIII* digested amplified PCR products of the genus *Bacillus* formed 2 experimental and 6 theoretical-*HaeIII*-ARDRA groups as showed in Fig. 2 and Table 4. The two experimental *HaeIII*-ARDRA clusters were as follows: cluster 1 which was consisted of solely *B. smithii* isolates and the cluster 2 which included the rest of the isolates including *B. pumilis*, *B. subtilis* and *B. licheniformis*. Cluster 1 differed from the second cluster by the presence of a 617 bp (1\textsuperscript{st}) and a 266 bp (3\textsuperscript{rd}) restriction fragment (Ba-H-1, Ba-H-2). Nevertheless, the three mentioned species in cluster 2 were indistinguishable from each other not only by the experimental...
but also the theoretical analyses. They displayed a dominant Ba-H-3 theoretical profile with exceptions of Ba-H-4 from E308, Ba-H-5 from E215 and Ba-H-6 from B51a. Furthermore, the restriction fragments of cluster 2 from the 650 bp (1st), the 358 bp (3rd) to the 147 bp (4th) differentiated this group from the B. smithii species.

The isolates from genus Brevibacillus were diverged into two HaeIII experimental-ARDRA clusters as presented in Fig. 2 and Table 4. There were also 4 theoretical ARDRA profiles with a frequently observed Br-H-1 profile on both of the clusters. Cluster 1 was comprised from B. thermoruber isolates and Cluster 2 from B. agri isolates. The distinctive restriction fragment of these clusters was the third restriction band. The molecular weight of the 3rd restriction fragment was calculated as 262 bp in B. thermoruber isolates, whereas this fragment was in 246 bp in B. agri isolates. Moreover, the D273a isolate displayed a P-H-1 theoretical-ARDRA profile with its closest relative: P. barengoltzii. Although the E302 and E3010c isolates belonging to the genus Thermoactinomyces displayed same experimental-ARDRA patterns, they differed in their theoretical profiles. While E3010c shared similar T-H-1 profile with its closest relate: T. vulgaris, E302 showed a distinct theoretical profile from E1010c and this type species (Table 4).

### Table 4. Some representative theoretical and experimental 16S rRNA gene HaeIII restriction fragments of isolates belonging to genera Bacillus, Brevibacillus, Paenibacillus and Thermoactinomyces

| Genus Bacillus | Bacteria | 16S rRNA gene sequence grouping | Experimental HaeIII | Theoretical HaeIII |
|----------------|----------|---------------------------------|---------------------|---------------------|
|                |          | Grouping | Fragments (bp) | Grouping | Fragments (bp) | Grouping |
|                |          |          | 1 | 2 | 3 | 4 | 5 | 6 | 7 |          | 1 | 2 | 3 | 4 | 5 |          |
| 1- B. smithii group | D362* | 657 494 330 | 1 | 446 237 | 1 | Ba-H-1 |
|                  | F92    | 645 499 332 | 1 | 563 458 175 | 77 | Ba-H-2d |
| 2- B. pumilis group | A381a | 653 494 360 130 70 | 2 | 596 456 253 | 1 | Ba-H-3d |
|                  | E308   | 650 494 355 138 70 | 2 | 597 458 2511 | 77 | Ba-H-3d |
| 3- B. subtilis group | E287   | 690 491 369 155 70 | 2 | 456 392 2581 | 203 | Ba-H-4 |
|                  | E215*  | 649 493 357 140 70 | 2 | 594 466 2481 | 77 | Ba-H-3d |
| 4- B. licheniformis group | E114 | 654 496 362 155 70 | 2 | 601 456 2511 | 77 | Ba-H-3d |
|                  | B51a*  | 654 496 362 155 70 | 2 | 242 225 213 | 77 | Ba-H-6 |

| Genus Brevibacillus | 1- B. thermoruber group | E165 | 634 478 263 143 | 1 | 591 456 1731 | 77 | Br-H-1d |
|                     | E292 | 639 486 262 149 | 1 | 456 428 1741 | 166 | Br-H-2 |
|                     | D295 | 639 486 259 148 | 1 | 456 401 191 176 | 77 | Br-H-3 |
| 2- B. agri group | E187 | 639 482 246 146 | 2 | 456 426 166 159 | 77 | Br-H-2 |
|                  | D505a | 634 478 245 141 | 2 | 590 443 1661 | 77 | Br-H-4 |
|                  | DSM 6348 | nd | 2 | 597 456 1981 | 133 | Br-H-4d |

| Genus Paenibacillus | P. barengoltzii group | E273a | 612 340 205 | 1 | 561 347 209 | 179 | 77 | P-H-1b |

| Genus Thermoactinomyces | T. vulgaris group | E3010c | 456 330 233 161 140 90 70 | 1 | 456 324 170 | 150 | 77 | T-H-1d |
|                         | E302* | 456 330 233 161 140 90 70 | 1 | 454 129 70 | 63 | 77 | T-H-2 |

(Abbreviations: *, strains having partial 16S rRNA gene sequences; bold fragment, the distinctive HaeIII restriction fragment; underlined fragment, the 3rd fragment of the 16S rRNA gene sequence; Ba-H-#, Baccilus-HaeIII-# theoretical group; Br-H-#, Brevibacillus-HaeIII-# theoretical group; P-H-#, Paenibacillus-HaeIII-# theoretical group; T-H-#, Thermoactinomyces-HaeIII-# theoretical group; d, the dominant theoretical profile among the distinct 16S rRNA gene groups; nd, not detected. The designation of the novel isolates were showed in bold character)
**TaqI-ARDRA profiles of the isolates**

The TaqI-ARDRA analyses of genus Bacillus revealed 3 experimental and 5 theoretical groups. Among the 3 experimental clusters derived from the cluster analyses of the isolates belonging to genus Bacillus, cluster 1 branched into two groups (Fig. 2, Table 5). One of which comprised all the B. smithii isolates and surprisingly the only member of the second group was A391a isolate which showed 96.7 % and 91.9 % 16S rRNA gene sequence similarities to type species *B. pumilis* and *B. smithii*, respectively. Not theoretical, but the experimental-ARDRA sequence similarities to type species isolate which showed 96.7 % and 91.9 % 16S rRNA gene similarity dendrogram in Fig. 2. *B. thermoruber* isolates from cluster 1 and *B. agri* isolates from cluster 2. Although theoretical group Br-T-1 was commonly observed in cluster 1, *B. agri* isolates displayed different theoretical groups not only from each other, but also from the type species. In addition, the presence of the 325 bp (3º) and 198 bp (5º) fragments in cluster 1 and the presence of a 511 bp (1º) restriction fragment in cluster 2 differed these groups from each other. Moreover, as observed in *Alu*-theoretical ARDRA profiles of the isolates from genera *Paenibacillus* and *Thermoactinomyces*, they showed similar profiles as their closely related type species (Table 5).

**Table 5.** Some representative theoretical and experimental 16S rRNA gene TaqI restriction fragments of isolates belonging to genera Bacillus, Brevibacillus, Paenibacillus and Thermoactinomyces

| 16S rRNA gene sequence grouping | Bacteria | Experimental Taq1 | Theoretical Taq1 | Grouping |
|---------------------------------|----------|------------------|------------------|----------|
| Genus Bacillus                  |          |                  |                  |          |
| 1- B. smithii group             |          |                  |                  |          |
| F92                             |          | 584 537 440 85   | 1 593 498 406    | Ba-T-1^d |
| 2- B. pumilis group             |          | 589 537 436 77   | 1 903^3 491      | Ba-T-2   |
| A391a                           | E308     | 961 385 215 70   | 2 904 358 135    | Ba-T-3^d |
| 3- B. subtilis group            |          |                  |                  |          |
| E215†                           |          | 521 415 373 208 70 | 3 397 224 127    | Ba-T-4^d |
| D311                           |          | 515 414 365 198 70 | 3 501 404 358    | Ba-T-4^d |
| 4- B. licheniformis group       |          |                  |                  |          |
| E114                            |          | 522 418 385 208 70 | 3 502 404 358    | Ba-T-4^d |
| B51a†                           |          | 523 415 385 209 70 | 3 490^1 259      | Ba-T-5   |
| DSM 13^7                        |          | 521 414 377 215 70 | 3 906 358 142    | Ba-T-3   |
| Genus Brevibacillus             |          |                  |                  |          |
| 1- B. thermoruber group         |          |                  |                  |          |
| D295                            |          | 422 354 329 216 197 | 75 1 407^3 310 288 | 201 183 | Br-T-1^d |
| C292                            |          | 421 354 325 213 198 | 75 1 514 405^3 284 | 183 Br-T-2 |
| E165                            |          | 422 354 325 216 195 | 75 1 492 404 284 | 201 Br-T-3 |
| 2- B. agri group               |          |                  |                  |          |
| E187                            |          | 511 400 354 209 70 | 2 556^3 320 286 | 210 Br-T-4 |
| D505a                           |          | 513 405 354 207 70 | 2 680 395 283    | Br-T-5   |
| DSM 6348^7                      |          | nd                | 2 498 395 356 201 | Q^d      |
| Genus Paenibacillus             |          |                  |                  |          |
| P. barengoltzi group            |          |                  |                  |          |
| D273a                           |          | 856 595 138      | 1 787^3 491 119  | P-T-1^d |
| Genus Thermoactinomyces         |          |                  |                  |          |
| T. vulgaris group               | E3010c   | 918 550          | 1 897^3 487      | T-T-1^d  |

(Abbreviations: , strains having partial 16S rRNA gene sequences; bold fragment, the distinctive TaqI restriction fragment; underlined fragment, the 3’ fragment of the 16S rRNA gene sequence; †, the 5’ fragment of the 16S rRNA gene sequence; Ba-T-#, Bacillus-TaqI-#d theoretical group; Br-T-#, Brevibacillus-TaqI-#d theoretical group; P-T-#, Paenibacillus-TaqI-#d theoretical group; T-T-#, Thermoactinomyces-TaqI-#d theoretical group; ^d, the dominant theoretical profile among the distinct 16S rRNA gene groups; nd, not detected. The designation of the novel isolates were showed in bold character)
**DISCUSSION**

With the rapid accumulation of 16S rRNA gene sequences in public databases, this technique has been widely used when designating the phylogenetic position of prokaryotic organisms and constitute the basis of the modern bacterial taxonomy (28). Comparative sequence analysis revealed that there were some limitations of this technique when determining the relationships of genetically closely related microorganisms at the species level (29). The others are the differences in sizes of sequenced 16S rRNA genes and also some technical and functional errors in sequences, which might contained the disappearance or appearance of one or more nucleotides, deposited in databases (25). Moreover, it was accepted that species showing 70 % or greater DNA-DNA homology usually have more than 97 % 16S rRNA gene sequence similarities. Thus, the DNA-DNA hybridization experiments still constitute the superior method when 16S rRNA gene sequences of the novel strains show 97 % or more similarity with its closest relatives (16, 28, 29).

In this study, the 115 endospore-forming bacilli, previously isolated from wide geothermal regions of Turkey, were mainly grouped into three according to their temperature requirements. Majority of the isolates were found to be thermophilic (74 %) as expected because of their hot sources of origin. The number of the isolates being facultative thermophilic and mesophilic were relatively low with 16 facultative thermophilic and 14 mesophilic strains. But as it is known, the members of Bacillus genetic group 1 to 6 belonging to the family Bacillaceae form a unique type of resting cell called endospore. Endospore formation, universally found in this group, is thought to be a strategy for survival in their habitats including the hot environments (5). Therefore, mostly the non-thermophilic, endospore-forming members of these geothermal habitats were selected for further studies.

The comparative sequence analyses based on the individual 16S rRNA gene sequence similarities revealed that the majority of mesophilic and facultative thermophilic isolates, which were presented in this study, were belonged to the genus Bacillus genetic groups 1 and 3. These bacterial isolates were identified as members of the genera Bacillus (16), Brevibacillus (13), Thermoactinomyces (2) and Paenibacillus (1). All of them were branched within these genetic groups except Thermoactinomyces isolates which formed a distinct cluster with thermophilic genera Anoxybacillus and Geobacillus from genetic group 5. Among the 16 isolates belonged to genus Bacillus, fourteen of them was able to cluster into four distinct lineages: in B. pumilis, B. licheniformis, B. subtilis and B. smithii groups with 6, 3, 3 and 2 isolates. However, isolates of A391a and B51a could not included any of the described type strains of genus Bacillus as concluded from their low level sequence similarity values to their closest relatives with similarities of 96.7 % to B. pumilis and 95.3 % to B. licheniformis, therefore they represented two novel species related with genus Bacillus. In addition, the rest of the isolates were found to be belonged to B. thermoruber (9), B. agri (3), T. vulgaris (2) and P. barengoltzii (1), except D295 isolate which displayed a 97.5 % borderline 16S rRNA gene sequence similarity to its closest relative B. thermoruber. Thus, as in the case of A391a and B51a isolates, the nearly complete sequence comparison of D265 isolate proposed that it represented a novel species among genus Brevibacillus, and these data will lead to their further genotypic and phenotypic analysis.

Moreover, DNA-directed genotypic fingerprinting methods such as amplified ribosomal DNA restriction analysis have been well-studied among the thermophilic, endospore-forming bacteria, and shown to be a valuable, easy and accurate technique for the identification of genera Bacillus and Geobacillus (3, 14, 20, 33, 35). In the previous studies, restriction endonucleases of AluI, CfoI, HaeIII, HinfI, MseI, RsaI, TaqI were used when genotyping and of those from enzymes, AluI and HaeIII were the most frequently used enzymes for ARDRA analyses of endospore-forming bacilli, as
they produced the highest number of differentiating bands (15). It is also known that rRNA genes are organized as a multigene family and expressed with a copy number from 1 to 15 (12). As there might be sequence heterogeneity among multiple 16S rRNA genes, this will probably affect the recognition sites of the restriction endonucleases. Consequently, it was recommended that the theoretically and experimentally obtained digestion profiles should be compared (3, 15).

On behalf of these explanations, the amplified 16S rRNA gene products of the isolates from genera Bacillus, Brevibacillus, Paenibacillus and Thermoactinomyces were subjected to both experimental and theoretical digestions with AluI, HaeIII and TaqI restriction enzymes. The AluI-, HaeIII- and TaqI-ARDRA profiles allowed us to distinguish all of the isolates and the reference strains, and the differentiating restriction bands were also determined. These results revealed that, AluI ARDRA patterns of isolates from B. smithii, B. pumilis, B. subtilis and B. licheniformis; HaeIII ARDRA pattern of isolates belonging to B. smithii and TaqI ARDRA patterns of B. smithii, B. pumilis and A391a isolate were all unique. Surprisingly, although the novel isolate A391a, of which its closest relative was determined as B. pumilis with a low sequence similarity, this isolate displayed a similar pattern with isolates from B. smithii group by means of its TaqI ARDRA pattern. Furthermore, all the AluI, HaeIII and TaqI restriction enzyme digestion patterns were successful in distinguishing the isolates from genus Brevibacillus into two species groups: B. agri and B. thermoruber. It is obvious that the potential of proposed AluI ARDRA technique is superior on ARDRA profiles obtained using HaeIII and TaqI due to the number of restriction fragments, especially when determining the genetic diversity of isolates from genus Bacillus. In addition, some differences in the theoretical and experimental ARDRA profiles can be explained by the size of our sequenced 16S rRNA genes and the ones published in databases. The other reason may also be some technical and functional errors in sequences, which might contain the disappearance or appearance of one or more nucleotides (25). This kind of ARDRA techniques was not always found useful when identifying genetically polymorphic groups of strains, for which DNA hybridization remains the needed method for identifying these closely related taxa at the species level (34). As a consequence, although there were some limitations, such as ARDRA analyses were carried out on conserved 16S rRNA genes, we were able to differentiate and cluster our isolates by using their ARDRA patterns. The ARDRA results also showed resemblance with the 16S rRNA gene sequence analyses. By ARDRA results, not only the discriminative restriction fragments of these isolates and type species were determined, but also the novelty of our A391a isolate could be demonstrated.

Consequently, the genetic diversity of isolates from genus Bacillus and Baccillus-related bacteria in geothermal areas of Turkey were presented, some of which are novel. Certain differentiating phenotypic characters of these isolates were studied and some of these bacilli which might have biotechnological potential in industrial applications, exhibited significant amount of halo zones in amylase, chitinase and protease assays, when compared with reference strains. Although majority of these isolates were lack of producing carbohydrate degrading enzymes, on the contrary they were found to be capable of producing protease enzymes notably in isolates belonging to B. subtilis and B. licheniformis. It was also concluded that the mesophilic and facultative thermophilic endospore-forming groups were able to live and shared the same extremely hot habitats with their thermophilic counterparts, on behalf of their endospore formation ability in order to survive in these environments. The reliability of species identification scheme including genus Bacillus, Brevibacillus, Paenibacillus and Thermoactinomyces of proposed ARDRA techniques were also proved in congruence with the phylogenetic analyses of the 16S rRNA gene sequences.
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