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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. lichenformis* (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 *Alu*I-, *Hae*III- and *Taq*I-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 reclassification as advances in molecular biology have revealed a high phylogenetic heterogeneity (5, 21). The genus *Bacillus*

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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 G*eobacillus* (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, Geobacillus, 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, Aydin (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 Izmir (Region D; 27º 09´ E, 38º 25´ N) provinces are in the Aegean Region, whereas Nevsehir (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 % K_2HPO_4 and 0.1 % KH_2PO_4, (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 °C 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**
A111[a], A181[b], A296 [a]	Omerbeyli, Germencik, Aydin, Turkey	3
A381a [a], A391a[a]	Yavuzkoy, Salavatli, Aydin, Turkey	2
B51a [b], B66[c], B91[a], B93[a]	Urganlı, Turgutlu, Manisa, Turkey	4
C83ca[c], C292[b]	Buharkent, Tekkehamam/Tekkekoy, Denizli, Turkey	2
D75a[a], D75b[a]	Balcova Geothermal Site, Izmir, Turkey	2
D45[c]	Seferhisar, Urkmez, Izmir, Turkey	1
D273a[a], D295[d], D311[c], D662b[b]	Seferhisar, Doganbey, Izmir, Turkey	4
D194a[b], D194b[b], D505a[b], D505b[b]	Dikili, Kaynarca, Kocaoba, İzmir, Turkey	4
D362[b]	Dikili, Zeytindalı, İzmir, Turkey	1
E114[b], E287[b], E302[a], E308[a], E3010c[a]	Altinsu, Kozakli, Nevsehir, Turkey	5
E165[a], E187[a], E215[a]	Baglica Kozakli, Nevsehir, Turkey	3
F92[a]	Kizilcahamam, Ankara, Turkey	1
	Total number of the bacterial isolates	32
Reference strains		
Bacillus amyloliquefaciens DSM7[T]	DSMZ	
Bacillus licheniformis DSM 13[T]	DSMZ	
Bacillus coagulans DSM 1[T]	DSMZ	
B. subtilis DSM 347[T]	DSMZ	

[a]; soil sample, [b]; sediment sample, [c]; water sample, [d]; 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 ºC for 24-72 h. All the incubation conditions were adjusted to 37 ºC, 45 ºC or 60 ºC 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 $MnSO_4.4H_2O$ (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 amylolytic (7). When determining α-glucosidase activity, a screening was carried out on MI plates by searching *para*-nitrophenol α-D-glucopyranoside (*p*NPG) activity on blotting filter paper as described previously (4). The paper disk was incubated at 40 ºC or 60 ºC 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)^p, A381a (FJ429999), A391a (FJ430001), B51a (FJ429572)^p, C83ca (FJ429573)^p, D75a (FJ430020), D194a (FJ430022), D311 (FJ430035), D362 (FJ429579)^p, E114 (FJ430054), E215 (FJ429588)^p, E287 (FJ430065), E308 (FJ430066), F92 (FJ430068), D273a (FJ430033), A181 (FJ429991), A296 (FJ429992), B66 (FJ430008), B91 (FJ430010), B93

(FJ430011), C292 (FJ430017), D45 (FJ430019), D295 (FJ430034), D505a (FJ430048), D662b (FJ430053), E165 (FJ430055), E187 (FJ430060), E302 (FJ429592)[p], E3010c (FJ430067), ([p]: the partial 16S rRNA gene sequences).

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 *Alu*I, *Hae*III and *Taq*I 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 ºC, T_{opt}: 30 - 40 ºC), the facultative thermophiles (25 - 60 ºC, T_{opt}: 40 - 50 ºC) and the thermopiles (40 - >70 ºC, T_{opt}: 50 - >70 ºC). 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 ºC. 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).

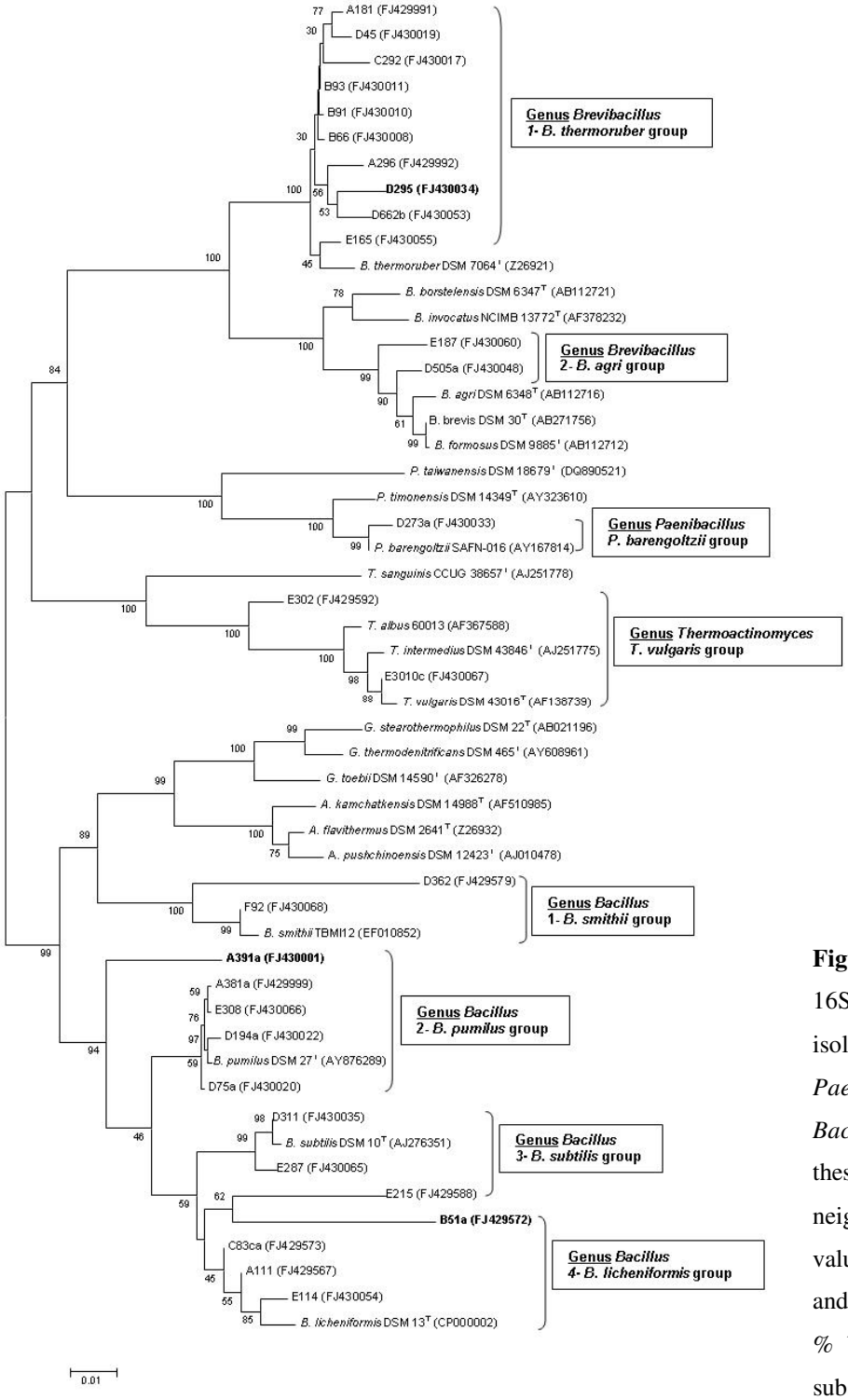

Figure 1. A phylogenetic tree based on the 16S rRNA gene sequences between isolates belonging to genus *Brevibacillus, Paenibacillus, Thermoactinomycetes, Bacillus* and the related members from these genera. The tree was generated by neighbour-joining method. Boostrap values (%) are based on 1000 replicates 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*, *Thermoactinomycetes* and *Paenibacillus*

As the isolated bacteria were originated from hot environments, representatives of the genera *Bacillus*, *Brevibacillus*, *Thermoactinomycetes* 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 reveled 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. pumilis* 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 *Thermoactinomycetes 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
		Isolates belonging to genus *Bacillus*	16
Brevibacillus	1- *B. thermoruber* group	97.5-99.1	10
	2-*B. agri* group	98.6-99.2	3
		Isolates belonging to genus *Brevibacillus*	13
Thermoactinomyces	1-*T. vulgaris* group	97.3-99.8	2
		Isolates belonging to genus *Thermoactinomyces*	2
Paenibacillus	1-*P. barengoltzii* group	99.8	1
		Isolates belonging to genus *Paenibacillus*	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_{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_{opt} = 55 °C), *B. licheniformis* (25 - 55 °C, T_{opt} = 37 °C) and *B. thermoruber* (35 - 58 °C, T_{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_{opt} = 30 °C), *B. pumilis* (20 - 45 °C, T_{opt} = 30 °C) and *B. agri* (20 - 40 °C, T_{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.

***Alu*I-ARDRA profiles of the isolates**

Distinctive ARDRA patterns were obtained after restriction with *Alu*I of the amplified 16S rRNA gene of

isolates from the genera *Bacillus*, *Brevibacillus*, *Thermoactinomycetes* 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 *Alu*I-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-Alu*I-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 (5^{th}) and 87 bp (6^{th}) fragments. Cluster 3 included isolates from *B. subtilis* which could be distinguished from the others by a 98 bp (5^{th}) restriction fragment (Ba-A-5, Ba-A-6). Cluster 4 contained isolates from *B. licheniformis* with a unique 825 bp (1^{st}) 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-*Alu*I-ARDRA profiles with their closely related type species (Table 3).

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

		Experimental *Alu*I							**Theoretical *Alu*I**							
16S rRNA gene sequence grouping	Bacteria	Fragments (bp)						Grouping	Fragments (bp)							Grouping
		1	2	3	4	5	6		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	451	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[d]
	E308	450	284	226	201	102	87	2	427	264	206	185	122	87	84	Ba-A-4[d]
3- *B. subtilis* group	E287	450	285	225	200	99		3	425	264	206	185	172	115		Ba-A-5[d]
	E215*	450	284	229	204	98		3	259	192	180	116†				Ba-A-6
4- *B. licheniformis* group	C83ca*	825	270	139	115			4	368	264	116†					Ba-A-7[d]
	B51a*	825	270	139	115			4	365	264	119†					Ba-A-7[d]
	E114	825	270	139	115			4	825	264	139	113				Ba-A-8
	SK13.02	825	270	139	115			4	429	264	206	185	172	129		Y
Genus *Brevibacillus*																
1- *B. thermoruber* group	C292	619	455	212	153			1	612	379	206	140				Br-A-1[d]
	D295	610	460	210	150			1	610	383	206	140				Br-A-1[d]
2- *B. agri* group	E187	447	418	245	214	167	79	2	394	381	217	174†	160			Br-A-2[d]
Genus *Paenibacillus*																
P. barengoltzii group	D273a	440	420	215	186	80		1	417	384	215	185	87	86		P-A-1[d]
Genus *Thermoactinomycetes*																
T. vulgaris group	E3010c	429	410	402	255	80		1	414	403	380	185†				T-A-1[d]

(Abbreviations: *, strains having partial 16S rRNA gene sequences; bold fragment, the distinctive *Alu*I restriction fragment; underlined fragment, the 3´ fragment of the 16S rRNA gene sequence; †, the 5´ fragment of the 16S rRNA gene sequence; Ba-A-#, *Bacillus-Alu*I-#nd theoretical group; Br-A-#, *Brevibacillus-Alu*I-#nd theoretical group; P-A-#, *Paenibacillus-Alu*I-#nd theoretical group; T-A-#, *Thermoactinomyces-Alu*I-#nd 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)

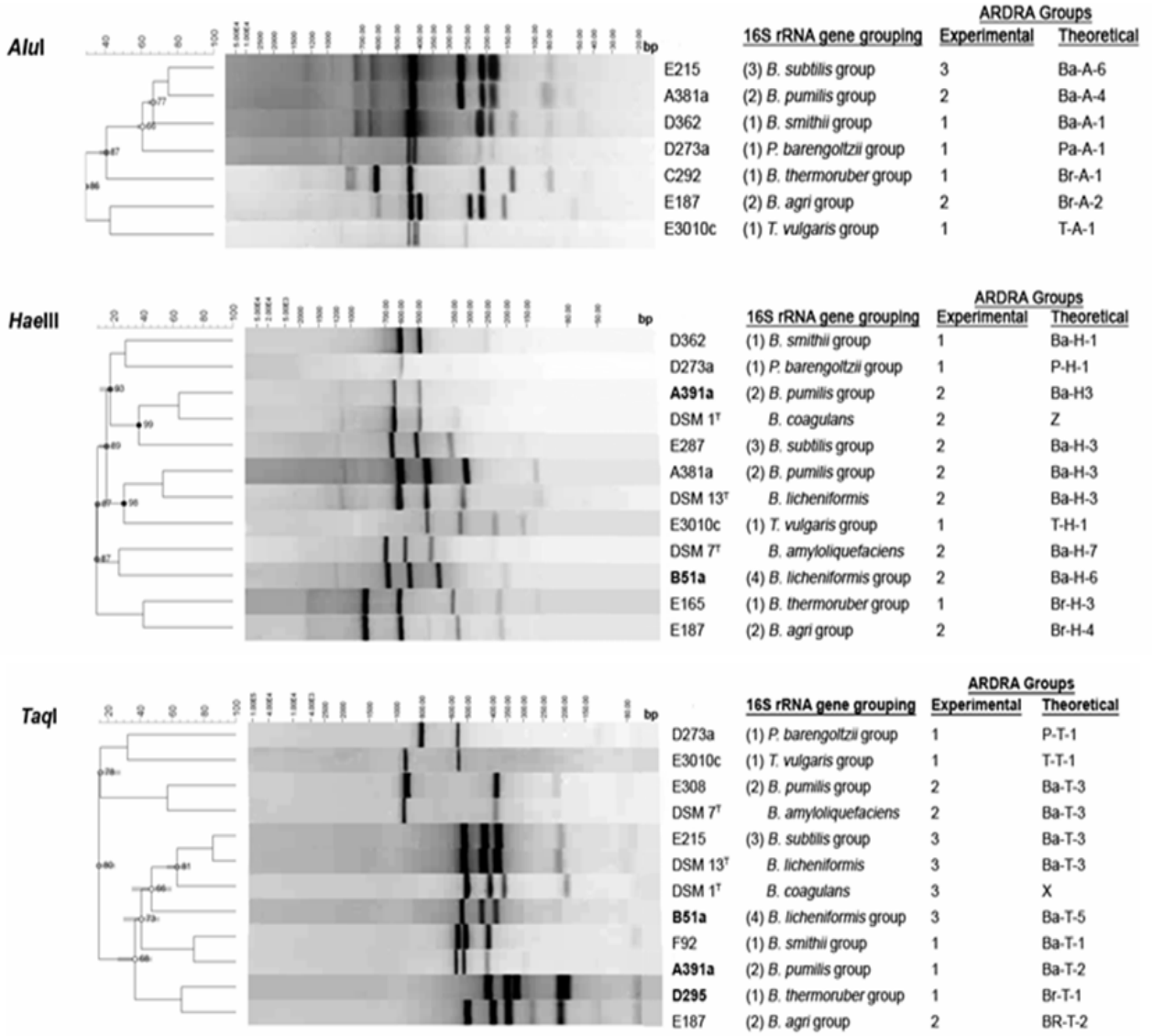

Figure 2. Cluster analysis of some representative digitized banding patterns, generated by restriction digestions with *Alu*I, *Hae*III and *Taq*I enzymes of the amplified 16S rRNA genes of isolates from genus *Brevibacillus, Paenibacillus, Thermoactinomycetes* and *Bacillus*. The dendrogram was constructed by using UPGMA, with correlation levels expressed as percentage values of the Dice coefficient. The 16S rRNA gene and the ARDRA groups derived from both experimental and theoretical restriction digestions were also indicated beside the designation of the isolate. The novel strains were written in bold character.

*Hae*III-ARDRA profiles of the isolates

The *Hae*III digested amplified PCR products of the genus *Bacillus* formed 2 experimental and 6 theoretical-*Hae*III-ARDRA groups as showed in Fig. 2 and Table 4. The two experimental *Hae*III-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 (1st) and a 266 bp (3rd) 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 *Hae*III 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 *Hae*III restriction fragments of isolates belonging to genera *Bacillus, Brevibacillus, Paenibacillus* and *Thermoactinomyces*

		Experimental *Hae*III								**Theoretical *Hae*III**					
16S rRNA gene sequence grouping	Bacteria	Fragments (bp)							Grouping	Fragments (bp)					Grouping
		1	2	3	4	5	6	7		1	2	3	4	5	
Genus *Bacillus*															
1- *B. smithii* group	D362*	657	494	330					1	446	237				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†			Ba-H-3d
	A391a	650	494	355	138	70			2	597	458	251†			Ba-H-3d
	E308	690	491	369	155	70			2	456	392	258†	203		Ba-H-4
3- *B. subtilis* group	E287	655	493	357	140	70			2	594	466	248†			Ba-H-3d
	E215*	649	493	368	149	70			2	435	246				Ba-H-5
4- *B. licheniformis* group	E114	658	495	372	162	70			2	601	456	251†			Ba-H-3d
	B51a*	654	496	362	155	70			2	242	225	213			Ba-H-6
Genus *Brevibacillus*															
1- *B. thermoruber* group	E165	634	478	263	143				1	591	456	173†			Br-H-1d
	C292	639	486	262	149				1	456	428	174†	166		Br-H-2
	D295	639	486	259	148				1	456	401	191	176†		Br-H-3
2- *B. agri* group	E187	639	482	246	146				2	456	428	166	159†		Br-H-2
	D505a	634	478	245	141				2	590	443	166†			Br-H-4
	DSM 6348T	nd							2	597	456	198†	133		Br-H-1d
Genus *Paenibacillus*															
P. barengoltzii group	D273a	612	340	205					1	561	347	209	179†	77	P-H-1d
Genus *Thermoactinomycetes*															
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†	30	T-H-2

(Abbreviations: *, strains having partial 16S rRNA gene sequences; bold fragment, the distinctive *Hae*III restriction fragment; underlined fragment, the 3´ fragment of the 16S rRNA gene sequence; †, the 5´ fragment of the 16S rRNA gene sequence; Ba-H-#, *Bacillus-Hae*III-#nd theoretical group; Br-H-#, *Brevibacillus-Hae*III-#nd theoretical group; P-H-#, *Paenibacillus-Hae*III-#nd theoretical group; T-H-#, *Thermoactinomyces-Hae*III-#nd 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)

***Taq*I-ARDRA profiles of the isolates**

The *Taq*I-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 profiles of *B. smithii* isolates (Ba-T-1) and A391a (Ba-T-2) were similar, thus A391 isolate was included to experimental cluster 1 along with other *B. smithii* isolates. The first (584 bp) and the third (440 bp) restriction fragments were the distinctive fragments of this cluster. Cluster 2 was composed of *B. pumilis* isolates containing a 961 bp (1st) distinctive restriction fragment and displaying the common Ba-T-3 theoretical-ARDRA profile. Finally, cluster 3 was branched into two *Bacillus* species: *B. subtilis* and *B. licheniformis* with a commonly observed Ba-A-4 theoretical profile. Cluster 3 differed from the other groups in the presence of 520 bp (1st) and 415 bp (2nd) restriction fragments.

The *Taq*I-ARDRA clustering also divided genus *Brevibacillus* into 2 experimental and 5 theoretical groups (Table 5). The experimental analyses revealed that isolates from genus *Brevibacillus* branched into two clusters as can be seen from the 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 (3rd) and 198 bp (5th) fragments in cluster 1 and the presence of a 511 bp (1st) restriction fragment in cluster 2 differed these groups from each other. Moreover, as observed in *Alu*I-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 *Taq*I restriction fragments of isolates belonging to genera *Bacillus, Brevibacillus, Paenibacillus* and *Thermoactinomyces*

		Experimental *Taq*I							Theoretical *Taq*I					
16S rRNA gene sequence grouping	Bacteria	Fragments (bp)						Grouping	Fragments (bp)					Grouping
		1	2	3	4	5	6		1	2	3	4	5	
Genus *Bacillus*														
1- *B. smithii* group	F92	584	537	440	85			1	593	498	406†			Ba-T-1d
2- *B. pumilis* group	A391a	589	537	436	77			1	903†	491				Ba-T-2
	E308	961	385	215	70			2	904	358	135			Ba-T-3d
3- *B. subtilis* group	E215*	521	415	373	208	70		3	397	224	127†			Ba-T-4d
	D311	515	414	365	198	70		3	501	404†	358	129		Ba-T-4d
4- *B. licheniformis* group	E114	522	418	385	208	70		3	502	404†	358	126		Ba-T-4d
	B51a*	523	415	385	209	70		3	490†	259				Ba-T-5
	DSM 13T	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†	310	288	201	183	Br-T-1d
	C292	421	354	325	213	198	75	1	514	405†	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†	320	286	210		Br-T-4
	D505a	513	405	354	207	70		2	680	395	283			Br-T-5
	DSM 6348T	nd						2	498	395	356	201		Qd
Genus *Paenibacillus*														
P. barengoltzii group	D273a	856	595	138				1	787†	491	119			P-T-1d
Genus *Thermoactinomycetes*														
T. vulgaris group	E3010c	918	550					1	897†	487				T-T-1d

(Abbreviations: *, strains having partial 16S rRNA gene sequences; bold fragment, the distinctive *Taq*I 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-Taq*I-#nd theoretical group; Br-T-#, *Brevibacillus-Taq*I-#nd theoretical group; P-T-#, *Paenibacillus-Taq*I-#nd theoretical group; T-T-#, *Thermoactinomyces-Taq*I-#nd 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 have 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), *Thermoactinomycetes* (2) and *Paenibacillus* (1). All of them were branched within these genetic groups except *Thermoactinomycetes* 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 *Alu*I, *Cfo*I, *Hae*III, *Hinf*I, *Mse*I, *Rsa*I, *Taq*I were used when genotyping and of those from enzymes, *Alu*I and *Hae*III 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 *Alu*I, *Hae*III and *Taq*I restriction enzymes. The *Alu*I-, *Hae*III- and *Taq*I-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, *Alu*I ARDRA patterns of isolates from *B. smithii*, *B. pumilis*, *B. subtilis* and *B. licheniformis*; *Hae*III ARDRA pattern of isolates belonging to *B. smithii* and *Taq*I 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 *Taq*I ARDRA pattern. Furthermore, all the *Alu*I, *Hae*III and *Taq*I 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 *Alu*I ARDRA technique is superior on ARDRA profiles obtained using *Hae*III and *Taq*I 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 *Bacillus*-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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