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Taxonomic Classification of *Anoxybacillus* Isolates from Geothermal Regions in Turkey by 16S rRNA Gene Sequences and ARDRA, ITS-PCR, *Rep*-PCR Analyses

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Abstract

A total of 115 endospore-forming bacilli were taken for 16S rRNA gene sequence analyses and clustered among 7 genera. In this paper, the most abundant thermophiles belonging to genus *Anoxybacillus* with its 53 isolates are presented. The *Anoxybacillus* species, some of which were producing biotechnologically valuable enzymes, mostly displayed amylolytic and glucosidic activities and the ability of carbohydrate degradation made them superior in number among the other bacilli in these extreme habitats. In comparative sequence analyses, similarities ranged from 91.1% to 99.9% between the isolates and the type strains. Isolates were clustered into eight phylogenetic lineages within the type strains of *A. kamchatkensis*, *A. flavithermus*, *A. kamchatchensis* subsp. *asaccharedens*, and *A. salavatliensis*. In addition, C161ab and A321 were proposed as novel species which displayed < 97.0% similarities to their closest relatives. Moreover, their individual *Alu*I, *Hae*III, and *Taq*I ARDRA restriction patterns, ITS-, $(GTG)_5$-, and BOX-PCR fingerprintings generated 27, 28, 31, 35, 40, and 41 clusters, respectively. The twelve type strains and 35 of the isolates showed unique distinctive patterns from all the others at least in two of these analyses. These phenotypic and genomic characters allowed us to differentiate their genotypic diversity from the reference strains.

Key words: *Anoxybacillus,* isolation, thermophilic, 16S rRNA gene, ARDRA, ITS- and *Rep*-PCR

Introduction

Although the genus *Anoxybacillus* contains the endospore-forming, thermophilic rod shaped bacteria which are close to the genus *Bacillus*, they have only been described recently by Pikuta *et al.* (Pikuta *et al*., 2000; 2003). Since then, the number of *Anoxybacillus* species of which this genus is comprised has rapidly increased and now contains the following nineteen validly described species and 3 subspecies: *Anoxybacillus pushchinoensis*, *Anoxybacillus flavithermus* (Pikuta *et al*., 2000), *Anoxybacillus gonensis* (Belduz *et al*., 2003), *Anoxybacillus contaminans* (De Clerck *et al*., 2004) *Anoxybacillus voinovskiensis* (Yumoto *et al*., 2004), *Anoxybacillus kestanbolensis*, *Anoxybacillus ayderensis* (Dulger *et al*., 2004), *Anoxybacillus kamchatkensis* (Kevbrin *et al*., 2005), *Anoxybacillus amylolyticus* (Poli *et al*., 2006), *Anoxybacillus rupiensis* (Derekova *et al*., 2007), *Anoxybacillus bogrovensis* (Atanassova *et al*., 2008), *Anoxybacillus kamchatkensis* subsp. *asaccharedens* (Gul-Guven *et al*., 2008), *Anoxybacillus thermarum* (Poli *et al*., 2009), *Anoxybacillus eryuanensis*, *Anoxybacillus tengchongensis* (Zhang *et al*., 2010), *Anoxybacillus salavatliensis* (Cihan *et al*., 2011), *Anoxybacillus mongoliensis* (Namsaraev *et al*., 2010), *Anoxybacillus flavithermus* subsp. *flavithermus*, *Anoxybacillus flavithermus* subsp. *yunnanensis* (Dai *et al*., 2011), *Anoxybacillus caldiproteolyticus* (Coorevits *et al*., 2012), *Anoxybacillus tepidamans* (Schäffer *et al*., 2004; Coorevits *et al*., 2012), and *Anoxybacillus kaynarcensis* (Inan *et al.,* 2012). Of those from these species, *A. kaynarcensis* and *A. kamchatkensis* subsp. *asaccharedens* are still not included in the validation list. In addition, most of the species belonging to genus *Anoxybacillus* were phylogenetically found to be a homogeneous group of thermophilic bacilli with high 16S rRNA gene sequence similarity values. Moreover the species from genus *Anoxybacillus* were aerotolerant anaerobes or facultative anaerobes as can be deduced from the genus name, but *Anoxybacillus* name means small rod living without oxygen (Pikuta *et al.*, 2000). Pikuta *et al.* (2000) first described the type strain of this genus as obligate anaerobe, later on Pikuta *et al.* (2003) presented a note to correct the description of the species *A. pushchinoensis* from obligate anaerobe to aerotolerant anaerobe and changed the description of the genus *Anoxybacillus* from obligate anaerobes to

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facultative anaerobes or aerotolerant anaerobes or facultative anaerobes.

Furthermore, isolation of novel thermophilic bacilli has received considerable attention since they include species of industrial, biotechnological and environmental interest. These thermozymes have been used in a number of industrial applications as they possess thermal stability to harsh industrial processes at high temperatures (Demirjian *et al*., 2001). Thermophilic bacilli are the natural source of many thermostable enzymes, and of those from thermophilic bacilli, some thermostable enzymes belonging to species from the genus *Anoxybacillus* such as amylase (Poli *et al*., 2006), glucosidase (Cihan *et al*., 2009), esterase (Colak *et al*., 2005), aldolase (Ertunga *et al*., 2007), proteinase (Lavrenteva *et al*., 2009) and xylanase (Kacagan *et al*., 2008) have been well characterized.

In this context, a polyphasic approach has been taking attention not only in microbial ecological researches in order to screen isolates producing novel thermostable enzymes that could suggest new applications (Derekova *et al*., 2008), but also in taxonomic studies when determining the phenotypic and genotypic diversity of these microorganisms, placing some of these bacilli in appropriate taxonomic levels, and also when characterizing novel species from these natural thermal habitats harbouring undiscovered microorganisms (Mora *et al*., 1998). This approach clusters a great number of similar bacteria belonging to the same genus and includes obtaining information about these clusters with definitive phenotypic and DNA-directed genotypic fingerprinting methods such as amplified ribosomal DNA restriction analysis (ARDRA) PCR reactions of intergenic transcribed spacers (ITS-PCR), and the repetitive extragenic palindromic elements (*Rep*-PCR) (Mora *et al*., 1998; Vaneechoutte *et al*., 1992; White *et al*., 1993).

Turkey is rich in geothermal sources which vary in typical temperatures and pH values and these environments have not yet been thoroughly studied. In the present study, the phylogenetic diversity of the isolates from genus *Anoxybacillus* was characterized and their taxonomic levels were determined by a polyphasic approach, all of which were isolated from geothermal areas in Turkey. Isolates were also screened for their amylolytic, glucosidic and proteolytic activities which might have biotechnological potential. The taxonomic data of these bacilli presented in this research were derived from the phenotypic characteristics, 16S rRNA gene sequences, ARDRA, ITS-PCR and *Rep*-PCR patterns. Therefore, this study is the first report which contains comprehensive phenotypic and genotypic data derived from a great number of *Anoxybacillus* isolates, all of which were obtained from wide geothermal regions in Turkey.

Experimental

Material and Methods

Sampling, isolation and growth conditions. During a previous polyphasic taxonomic study, more than five hundred thermophilic bacilli were isolated from different geothermal regions of Turkey (Coleri *et al*., 2009). In this study, 115 of the former isolates were randomly selected and taken into 16S rRNA gene sequence analyses and of those from the isolates only species belonging to genus *Anoxybacillus* are presented in the paper. The sampling, isolation and growth conditions of the isolates used in this study were as follows: A total of 108 samples including water (10), sediment (40), soil (55), stone (1) and tree branch (2) specimens were collected aseptically from 10 hot springs and 9 high-temperature well pipelines located in two geographically separated areas in Turkey: Aegean Region and Middle Anatolian Region. Of those geothermal regions, Aydin (Region A; 27°51' E, 37°51' N), Denizli (Region C; 29°06' E, 37°46' N) and Izmir (Region D; 27°09' E, 38°25' N) provinces are in the Aegean Region, whereas Nevsehir (Region E; 34°43' E, 38°38' N) and Ankara (Region F; 32°52' E, 39°56' N) provinces are located in the Middle Anatolian Region of Turkey. The water temperature and pH of these geothermal areas were between 50–100°C and 6.0–9.0, respectively.

One ml water and sediment sample or approximately 1 g soil, stone, and tree branch sample from each place was incubated in 5 ml of the MI medium containing 1% soluble starch (pH 7.0) at 60°C with 250 rpm 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 (Coleri *et al*., 2009; Suzuki *et al*., 1976). The turbid enrichments were streaked on plates of MI containing 3% agar and incubated aerobically at 60°C for 24–48 h. The single colonies having different colony morphology were then picked and sub-cultured at least three times until a pure culture was obtained. The cell morphologies of the isolates were also examined by phase contrast microscopic observation. All of the isolates were routinely maintained at 4°C on MI agar slants and stored at –80°C 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 53 isolates, their geothermal areas of origin and the reference strains used in this study are presented in Table I.

Morphologic and physiologic characterization. MI medium was preferred to Nutrient Agar in general incubation and morphological studies as it enabled a better growth of all these isolates aerobically or anaerobically

Table I
Diversity and origin of the *Anoxybacillus* isolates from different geothermal regions of Turkey and the reference strains used in this study.

Bacterial isolates	Origin	Number of bacteria
A321[a], A3210[a]	Omerbeyli, Germencik, Aydin, Turkey	2
A351a[b], A352b[b], A371[a], A402b[a], A414[a]	Yavuzkoy, Salavatli, Aydin, Turkey	5
C161ab[a], C163a[a], C245[a]	Buharkent,Tekkehamam/Tekkekoy, Denizli, Turkey	3
D36[b], D98a[a]	Balcova Geothermal Site, Izmir, Turkey	2
D52b[b]	Seferhisar, Urkmez, Izmir, Turkey	1
D202a[b], D203b[b], D204[b], D205[b], D211[c], D213[c], D214[c], D221a[c], D222b[c], D232a[b], D242b[c], D243[c], D455[b], D463[b], D486[b], D487[b], 503[b], D591[a], D594[a]	Dikili, Kaynarca, Kocaoba, İzmir, Turkey	19
D371a[b], D376b[b], D392[a], D394[a], D404[a], D433a[a]	Dikili, Zeytindalı, İzmir, Turkey	6
D401a[a]	Dikili, Camur Hot Spring, Izmir, Turkey	1
E123[b], E237[b], E243[b], E272[b], E331[d]	Altinsu, Kozakli, Nevsehir, Turkey	5
E183[a], E184aa[a], E184ab[a], E184b[a], E187[a], E206a[a], E206b[a], E208a[a], E208b[a]	Baglica Kozakli, Nevsehir, Turkey	8
F81[e]	Kizilcahamam, Ankara, Turkey	1
Total number of the bacterial isolates		**53**
Reference strains		
Anoxybacillus flavithermus DSM 2641^T	Kindly provided by Prof. A. O. Belduz	
Anoxybacillus kestanbolensis NCIB 13971^T	Kindly provided by Prof. A. O. Belduz	
Anoxybacillus gonensis NCIB 13933^T	Kindly provided by Prof. A. O. Belduz	
Anoxybacillus ayderensis NCIB 13972^T	Kindly provided by Prof. A. O. Belduz	
Anoxybacillus pushchinoensis DSM 12423^T	DSMZ	
Anoxybacillus thermarum DSM 17141^T	DSMZ	
Anoxybacillus kamchatkensis DSM 14988^T	DSMZ	
Anoxybacillus amylolyticus DSM 15939^T	DSMZ	
Anoxybacillus rupiensis DSM 17127^T	DSMZ	
Anoxybacillus voinovskiensis DSM 17075^T	DSMZ	
Anoxybacillus tepidamans DSM 16325^T	DSMZ	
Anoxybacillus thermarum DSM 17141^T	DSMZ	
Anoxybacillus salavatliensis DSM 22626^T	From our collection	
Total number of the reference strains		**13**

[a] – soil sample, [b] – sediment sample, [c] – water sample, [d] – branch of a tree, [e] – stone sample.

on plates. Thus, the temperature range was determined by incubating the strains in MI at temperatures from 20 to 80°C for 24–72 h. The cell morphology, motility and spore formation were observed with freshly prepared wet mounts using phase-contrast microscopy. The active cultures grown in MI Broth at 60°C under shaking for 18–24 h were used when describing the cell morphology and motility. The formation of spores was also tested by using MI broth cultures of 18–48 h supplemented with 5 mgL^{-1} $MnSO_4 \cdot 4H_2O$ (Claus and Berkeley, 1986).

The colony morphologies were determined using cultures grown aerobically for all the isolates or anaerobically for *A. pushchinoensis* on MI plates (supplemented with 3% agar) at 60°C for 18–24 h. Gram staining, oxidase and catalase activities were carried out by the methods of Claus and Berkeley as described previously (Claus and Berkeley, 1986). Nine reference strains were used as control groups in all the phenotypic and genotypic characterization tests. All the phenotypic characters were carried out in triplicates.

Enzyme assays. All of the isolates were screened for their amylase, α-glucosidase and protease activities qualitatively on agar plates. Amylolytic activity was tested on MI agar plates after incubation for 48 h. Then the plates were treated with iodine solution (0.2% I_2 in 2% KI) and isolates having starch digestion zones around their colonies were determined as amylolytic (Coleri *et al.*, 2009). When determining α-glucosidase activity, screening was carried out on MI plates by searching *para*-nitrophenol α-D-glucopyranoside (*p*NPG) activity on blotting filter paper as described

previously (Cihan *et al*., 2011). The paper disk was incubated at 60°C and the yellow colour formation, the colour 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 (Tekin *et al*., 2012). 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 colour formation were also measured, confirmed and compared with the reference strains which were able to produce these enzymes.

16S rRNA gene amplification and sequencing analyses. Genomic DNA was extracted from the cultures growing on MI medium for 18 h at 60°C 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 (Kuisiene *et al*., 2002). The amplification products were purified from agarose gel using Gel Extraction Kit (Omega Ezna). The 1000–1500 bp sequences of the PCR-amplified 16S rRNA genes were determined by using ABI 3100 gene sequencer with Bigdye cycle sequencing kit. In the phylogenetic analysis, homology search was carried out using both the basic BLASTN search program at the NCBI web-site and web-based public EzTaxon database (http://eztaxon-e.ezbiocloud.net/), (Kim *et al*., 2012). Phylogenetic analyses were performed using the maximum-likelihood and neighbour-joining methods with bootstrap values based on 1000 replications. The phylogenetic tree (Saitou and Nei, 1987) was constructed with the MEGA package version 4 (Tamura *et al*., 2007) and the nucleotide substitution distances were estimated by Jukes-Cantor model (Jukes *et al*., 1969). As the phylogenetic analyses derived from neighbour-joining method were in congruence with those obtained using the maximum-likelihood algorithm, only the data obtained from the neighbour-joining method is presented in the study.

Amplified ribosomal DNA restriction analysis of 16S rRNA gene. 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 for 1.5 h (Caccamo *et al*., 2001). The individual *Alu*I, *Hae*III and *Taq*I ARDRA patterns were analyzed by the GelCompar II software packages (Applied Maths, Belgium). The experimental restriction fragments higher than 45 bp were included in the statistical analysis. Similarities of the digitized profiles were calculated using Dice correlation and an average linkage (UPGMA) dendrogram was obtained for all the restriction analyses. All the restriction analyses and their agarose gel electrophoresis were carried out in triplicates. In addition to experimental restriction analyses, the theoretical *Alu*I, *Hae*III and *Taq*I restriction mapping of the analyzed 16S rRNA gene sequences were also carried out by using an online restriction mapping service (http://restrictionmapper.org/). As the experimental results were in relation with theoretical restriction mapping, only the experimental data is presented in this paper.

PCR based fingerprinting analyses of *Rep* elements and intergenic 16S-23S rRNA gene. Repetitive Elements-PCR (*Rep*-PCR) genomic fingerprintings were performed with the $(GTG)_5$ and BOXA1R primers using the PCR conditions that were described by Versalovic *et al.* (1994). Primer sets S-D-Bact-1494-a-S-20 and L-D-Bact-0035-a-A-15 were used for the amplification of intergenic transcribed spacers (ITS) between 16S and 23S rRNA genes and PCR conditions were adjusted according to Daffonchio *et al.* (2003). The PCR products electrophoresed in a 1.5% agarose gel, using 1 X TBE buffer at 120 V for 4 h. In the statistical analysis, triplicate individual ITS-PCR, BOXA1 and $(GTG)_5$-PCR fingerprintings were analyzed by the GelCompar II software packages (Applied Maths, Belgium). Similarities of the digitized profiles were calculated using Dice correlation and an average linkage (UPGMA) dendrogram was obtained.

Finally, the individually examined all the ARDRA analyses and the ITS-, $(GTG)_5$- and BOX-PCR fingerprintings were then taken into a cumulative cluster analysis which combined all these tests in a dendrogram by using the GelCompar II software packages. This combined results containing dendrogram was presented in this paper, instead of showing all the six individual cluster analyses of these fingerprinting tests. In clustering analyses according to the presence or absence of DNA bands and also to their densities, the similarity limits of 16.7, 33.34, 50.0, 66.7 and 83.4% values were used with GelCompar II software. In these context, the bacteria displaying 0–16.7% similarities were denoted as having unique distinctive profiles, the ones having similarities between 75.0% and 99.9% were determined as showing similar profiles and the ones with 100% similarity were implied as displaying the same profiles.

GenBank Accession Numbers. The 53 16S rRNA gene sequences presented in this study were obtained from the following *Anoxybacillus* isolates: A321 (FJ429993), A3210 (FJ429569), A351a (FJ429995), A352b (FJ429996), A371 (FJ430000), A402b (FJ430004), A414 (FJ430007), C161ab (FJ430012), C163a (FJ430013), C245 (FJ430016), D36 (FJ430018), D98a (FJ430021), D202a (FJ430024), D203b

(FJ430025), D204 (FJ430026), D205 (FJ430027), D211 (FJ430028), D213 (FJ430029), D214 (FJ430030), D221a (FJ430031), D222b (FJ429576), D232a (FJ429577), D242b (FJ429578), D243 (FJ430032), D371a (FJ429580), D376b (FJ430036), D392 (FJ430037), D394 (FJ429581), D401a (FJ430038), D404 (FJ430039), D433a (FJ430041), D455 (FJ429582), D463 (FJ430042), D486 (FJ430043), D487 (FJ430044), D503 (FJ430046), D52b (FJ429575), D591 (FJ430049), D594 (FJ429583), E123 (FJ429584), E183 (FJ430058), E184aa (FJ430059), E184ab (FJ429585), E184b (FJ429586), E206a (FJ430061), E206b (FJ430062), E208a (FJ430063), E208b (FJ429587), E237 (FJ429589), E243 (FJ430064), E272 (FJ429591), E331 (FJ429593), F81 (FJ429595).

Results

Selection of *Anoxybacillus* isolates according to the 16S rRNA gene sequence analyses. The 16S rRNA gene sequences of the 115 aerobic and endospore-forming isolates were analyzed in order to determine their phylogenetic position and to avoid the repeated examination in the same bacterial taxon. All the isolates 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 identified isolates from geothermal regions of Turkey 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 one hundred and fifteen of them were clustered among the 7 genera: *Anoxybacillus* (53 isolates), *Bacillus* (14 isolates), *Brevibacillus* (12 isolates), *Geobacillus* (26 isolates), *Aeribacillus* (4 isolates), *Paenibacillus* (1 isolates), and *Thermoactinomycetes* (2 isolates).

The *Anoxybacillus* isolates used in this study were isolated and purified from 19 geothermal sampling stations located in the Aegean Region and Middle Anatolian Region in Turkey. As indicated above, representatives of the genus *Anoxybacillus* were predominated among our isolates and 45% of the isolates were found to be belonged to this genus according to the 16S rRNA gene sequence analysis. The 16S rRNA gene sequence data of 53 *Anoxybacillus* isolates have been deposited in the GenBank databases and their accession numbers in relation to the isolates were given in the phylogenetic tree which was obtained using the neighbor-joining method (Fig. 1).

The phylogenetic diversity of *Anoxybacillus i*solates. The 16S rRNA gene sequence analyses in this study contains not only the phylogenetic diversity of our isolates, but also all the described *Anoxybacillus* species up to now. Thus, all the nineteen described type strains and 3 subspecies from genus *Anoxybacillus* were taken into comparative sequence analysis as presented in Fig. 1. In comparison analyses, all the members of genus *Anoxybacillus* listed in the introduction section, shared 16S rRNA gene sequence similarities from 93.8 to 99.9%. Furthermore, species of *A. flavithermus* DSM 2641^T, *A. salavatliensis* DSM 22626^T, *A. gonensis* NCIMB 13933^T, *A. ayderensis* NCIMB 13972^T, *A. kestanbolensis* NCIB 13971^T, *A. thermarum* DSM 17141^T, *A. kamchatkensis* DSM 14988^T, *A. kamchatkensis* subsp. *asaccharedens* DSM 18475^T, *A. eryuanensis* KCTC 13720^T, *A. tengchongensis* KCTC 13721^T, *A. pushchinoensis* DSM 12423^T, *A. mongoliensis* DSM 19169^T, *A. flavithermus* subsp. *flavithermus* BCRC 17265^T, *A. flavithermus* subsp. *yunnanensis* KCTC 13759^T, and *A. kaynarcensis* DSM 21706^T showed 16S rRNA gene sequence similarities from 97.4% to 99.9%. These strains were genetically found to be very homogeneous and be able to be differed from each other only by DNA-DNA hybridization analyses. Moreover, the rest of the *Anoxybacillus* species such as *A. bogrovensis* DSM 17956^T, *A. rupiensis* DSM 17127^T, *A. contaminans* DSM 15866^T, *A. voinovskiensis* DSM 17075^T, *A. amylolyticus* DSM 15939^T, *A. tepidamans* DSM 16325^T and *A. caldiproteolyticus* DSM 15730^T displayed a more heterogenic sequence homology with each other (from 93.9% to 99.6%) and formed distinct clusters in sequence comparisons.

According to the 16S rRNA gene sequence analysis, 53 of the isolates were found to belong to the genus *Anoxybacillus* as shown in the phylogenetic tree in Fig. 1. The sequence similarity values determined between the *Anoxybacillus* isolates and the recognized *Anoxybacillus* type strains were ranged from 91.1% to 99.9%. In addition, the sequence similarity values from 90.8% to 99.9% were determined between the *Anoxybacillus* isolates. Although most of the isolates had sequence similarity values higher than 97.0% to more than one closest relatives, the sequence comparison analyses given below were determined according to their most closely related type strains. According to these results, *Anoxybacillus* isolates branched within the phylogenetically homogenous *Anoxybacillus* type strains mentioned before. The clusters obtained from the 16S rRNA gene sequence analyses, the similarity values of the *Anoxybacillus* isolates to their closest relative(s) and content of these clusters were also presented in Table II.

The most abundant species was *A. salavatliensis* and this species was diverted into two groups. Cluster 1 was consisted of 8 isolates with sequence similarity values of 96.0–98.1% to *A. salavatliensis*, but although A321 isolate was branched within the *A. salavatliensis*

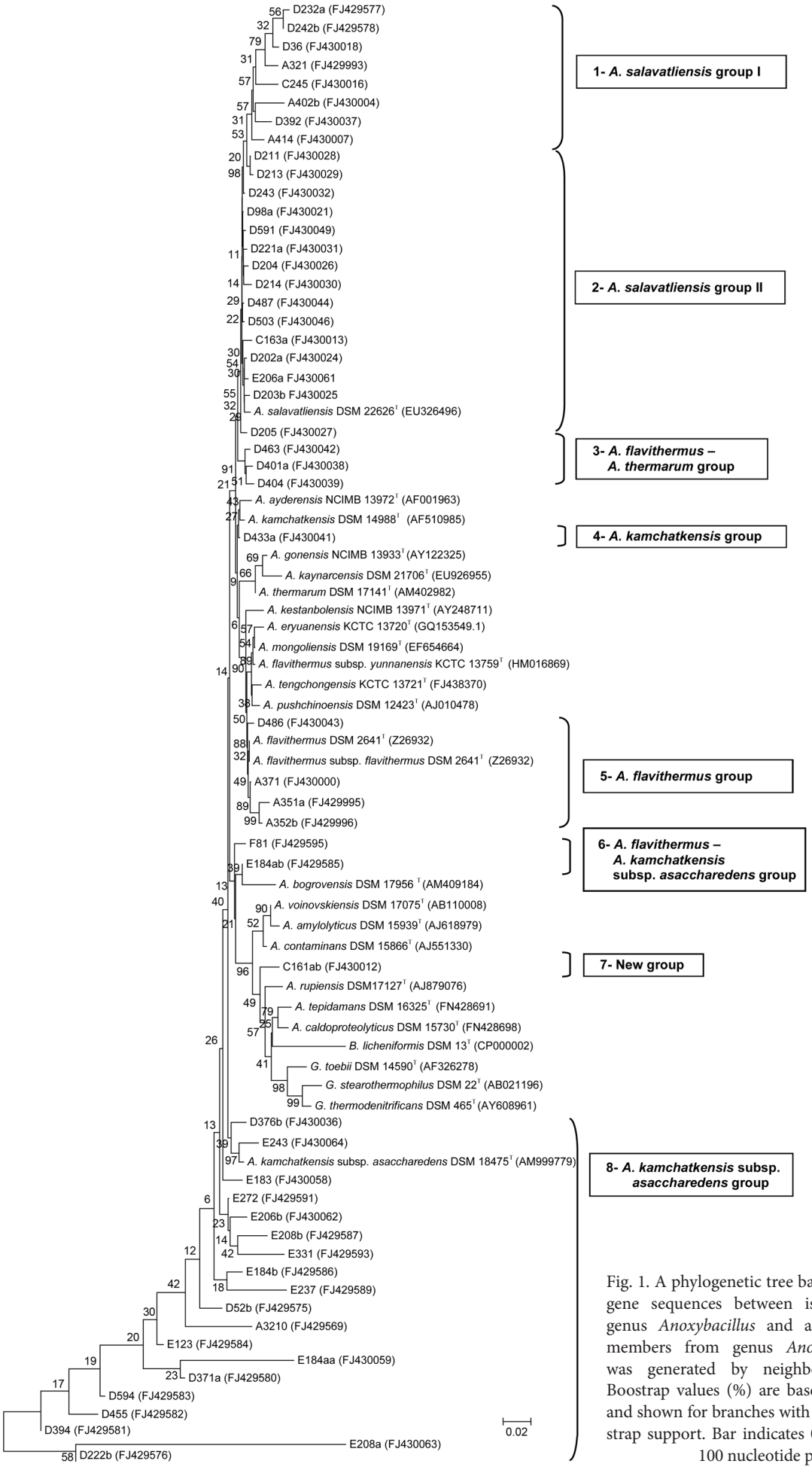


Fig. 1. A phylogenetic tree based on the 16S rRNA gene sequences between isolates belonging to genus *Anoxybacillus* and all the representative members from genus *Anoxybacillus*. The tree was generated by neighbour-joining method. Boostrap values (%) are based on 1000 replicates and shown for branches with more than 10% bootstrap support. Bar indicates 0.02 substitutions per 100 nucleotide positions.

Table II
The 16S rRNA gene sequence similarities of the 53 *Anoxybacillus* isolates to their closest relative(s)

According to 16S rRNA gene sequences			
The closest relative(s)		Similarities to the closest relative(s)	Contents of the phylogenetic clusters
1	*A. salavatliensis* DSM 22626^T	96.0–98.1%	D232a, D242b, A414, D392, C245, A402b, D36, A321
2	*A. salavatliensis* DSM 22626^T	98.5–99.8%	D204, D221a, D98a, D202a, D243, D487, E208a, D203b, D503, D591, D205, C163a, D211, D213, D214
3	*A. flavithermus* DSM 2641^T	98.8–98.9%	D463, D401, D404
	A. thermarum DSM 17141^T	98.6–98.8%	
4	*A. kamchatkensis* DSM 14988^T	99.6%	D433a
5	*A. flavithermus* DSM 2641^T	98.5–99.7%	A371, D486, A352b, A351a
6	*A. flavithermus* DSM 2641^T	98.2–99.3%	E184ab, F81
	A. kamchatkensis subsp. *asaccharedens* DSM 18475^T	98.4–99.5%	
7	*A. rupiensis* DSM17127^T *A. voinovskiensis* DSM 17075^T *A. caldoproteolyticus* DSM 15730^T	96.8–96.9% to all	C161ab
8	*A. kamchatkensis* subsp. *asaccharedens* DSM 18475^T	97.2–99.7%	E208a, E183, E184b , E206b, E243, D222b, E272, D52b, E123, E237, E208b, E184aa, E331, D594, E123, A3210, D455, D394

Cluster 1, it was not belonged to this type strains as it displayed a similarity value lower than 97%. A321 isolate displayed low similarity values solely to *A. salavatliensis* (96.0%), *A. kamchatkensis* (95.8%), *A. kamchatkensis* subsp. *asaccharedens* (95.4%) and *A. flavithermus* (95.3%) among all the described *Anoxybacillus* type strains. In addition, A321 displayed sequence similarities mostly to D36 (97.1%), D232a (98.6%) and D242b (98.6%) from other non-reference isolates in Cluster 1.

A. salavatliensis Cluster 2 comprised fifteen isolates having more genetic homogeneity not only with each other, but also with their closest relative. The closest relative(s) of the Clusters 3, 4, 5 and 6 were *A. flavithermus – A. thermarum*, *A. kamchatkensis*, *A. flavithermus*, and *A. flavithermus – A. kamchatkensis* subsp. *asaccharedens* containing 3, 1, 4 and 2 isolates, respectively.

The Cluster 7 contained only the isolate C161ab, which showed lower similarities than 97.0% to only *A. rupiensis* (96.9%), *A. voinovskiensis* (96.9%), and *A. caldoproteolyticus* (96.8%) from the all described *Anoxybacillus* type strains. Moreover, C161ab only showed higher than 97% sequence similarities only to E184ab (98.2%) and F81 (97.1%) among the other *Anoxybacillus* isolates. The 19 isolates comprising Cluster 8 distinguished from all these clusters having sequence similarities between 97.2–99.7% to their closest relatives *A. kamchatkensis* subsp. *asaccharedens*. Among the isolates in these eight clusters, A321 and C161ab represented potentially novel species, sharing less than 97.0% sequence similarity to their closest relatives.

Phenotypic characteristics of the isolates from genus *Anoxybacillus*. Colonies of all the *Anoxybacillus* isolates and the reference strains used were usually round and had a yellow pigmentation from pale yellow, yellow or orange yellow as a common character, when incubated on MI Agar plates, except D433a, C161ab and A371 isolates having cream colour colonies. Microscopic observations showed that their cells were mostly Gram-positive staining, motile (except *A. pushionensis*) and straight rods. Spore formation became detectable after 48–72 h incubation periods in liquid media. Their cells formed terminal, ellipsoidal to oval or round endospores in a swollen or non swollen sporangia.

The *Anoxybacillus* isolates and the reference strains were all thermophilic within the growth range between 30°C and 73°C with an optimal temperature value between 50 to 65°C. The isolates and the reference strains gave catalase positive reactions instead of *A. kamchatkensis* and *A. pushionensis* type strains, and C161ab isolates. Most of the isolates were able to grow anaerobically, but were facultative anaerobes, except *A. pushionensis*. In addition, *A. rupiensis* could only grow strict aerobically. The oxidase reaction varied relationally to the isolate and type strains.

Starch utilization was found to be a dominant character among genus *Anoxbacillus* except *A. kamchatkensis*, *A. thermarum*, and D392. Presence of carbohydrate degrading enzymes such as α-amylase and α-glucosidase was also a common characteristic of isolates and strains from genus *Anoxybacillus*, instead of *A. kamchatkensis* and *A. thermarum* type strains and isolates of D392 and E123. Furthermore, some of the isolates, which thought to have biotechnological potential in industrial applications, exhibited significant

Table III
Number of clusters of *Anoxybacillus* isolates and reference strains obtained from individual *Alu*I, *Hae*III and *Taq*I ARDRA profiles of 16S rRNA genes and ITS-, BOX- and GTG-PCR DNA fingerprintings

Number of	ARDRA profiles of 16S rRNA gene			ITS-PCR profiles	*Rep*-PCR profiles	
	*Alu*I	*Hae*III	*Taq*I		BOX-PCR	GTG-PCR
Standard clusters	8	11	8	12	12	12
Isolate clusters	20	16	23	23	29	28
Total clusters	28	27	31	35	41	40

amount of halo zones in amylase assay or produced more yellow colour in α-glucosidase assay, when compared with reference strains and the other isolates. Of those from amylolytic and glucosidic isolates, D323a, D98a, D404, E184aa, E184ab and D222b (6 isolates) had significant amount of amylolytic activity, whereas A414, D211, D213, D202a, D214, D204, E206, A351a, A352b, D3756b, A3210, E272, E208b, E331 and E206b (15 isolates) were capable of producing high levels of α-glucosidase. In addition, only 10 of 53 *Anoxybacillus* isolates could able to produced halo zones around their colonies in protease assay.

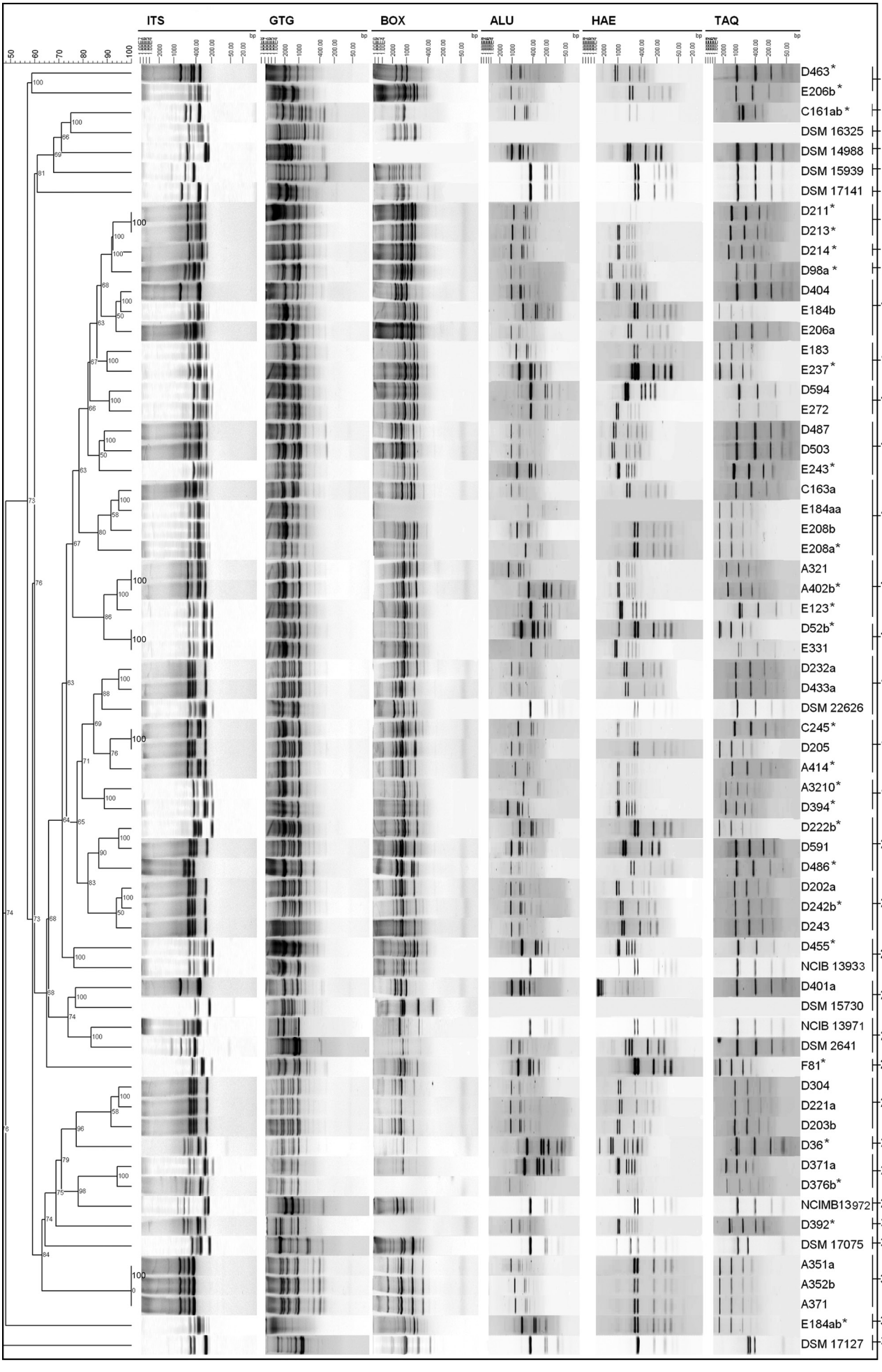
***Alu*I, *Hae*III and *Taq*I-ARDRA analyses of the *Anoxybacillus* isolates.** The amplified PCR products of the isolates from genus *Anoxybacillus* were subjected to digestion with *Alu*I, *Hae*III and *Taq*I restriction enzymes in comparison with reference strains. The individual *Alu*I-, *Hae*III- and *Taq*I-ARDRA cluster analyses of the digitized banding patterns derived from 53 *Anoxybacillus* isolates and 12 reference strains can be seen in Fig. 2. The number of clusters obtained from these three ARDRA profiles were shown in Table III, and the content of these individual clusters were indicated in Table IV. The isolates having distinctive patterns in these analyses were also indicated with "*" in Table IV.

In individual cluster analysis based on the *Alu*I-, *Hae*III- and *Taq*I-ARDRA profiles of the amplified 16S rRNA genes, totally 28, 27 and 31 clusters were observed when the presence or absence of the restriction fragments and also the density of the DNA bands were considered. In *Alu*I-ARDRA analysis, the 12 reference type strains were grouped into 8 clusters and in one of these clusters, *A. amyloyticus*, *A. thermarum*, *A. salavatliensis*, *A. gonensis* and *A. ayderensis* type strains formed the same patterns with each other. In addition, only seven of the isolates displayed distinctive patterns from all the isolates and type strains used. In *Hae*III- and *Taq*I-ARDRA analyses, these reference strains formed 11 to 8 clusters, respectively. While the reference strains of *A. gonensis* and *A. rupiensis* displayed the same *Hae*III-ARDRA patterns, in the case of *Taq*I-ARDRA analysis *A. amylolyticus* – *A. thermarum*, *A. ayderensis* – *A. kestanbolensis* and *A. flavithermus* – *A. kamchatkensis* – *A. gonensis* patterns matched with each other and these type strains grouped within three clusters. Beside these findings, 5 to 11 isolates showed unique patterns from all the isolates and reference strains used in *Hae*III- and *Taq*I-ARDRA analyses, respectively. According to these individual cluster analysis of the ARDRA tests, the *Hae*III-ARDRA analysis was found to be superior on the other restriction enzyme digestions for differentiating the reference strains within 11 unique clusters, whereas the number of profiles obtained from *Taq*I-ARDRA analyses was higher than the other restriction patterns, which allowed us to show the difference among all the isolates and reference strains.

The *Rep*-PCR and ITS-PCR fingerprintings of the *Anoxybacillus* isolates. All the *Anoxybacillus* isolates and their related type strains were subjected to the *Rep*-PCR and ITS fingerprinting analyses. While these fingerprinting results can be seen in Fig. 2, the numbers of the individual clusters and their contents are also presented in Table III and IV. In the individual cluster analyses of the *Rep*-PCR containing $(GTG)_5$ and BOX-PCR fingerprintings, totally 40 to 41 clusters were obtained and any of the isolates showed the same profile with the reference strains. Furthermore, 13 isolates in $(GTG)_5$-PCR and 12 isolates in BOX-PCR showed unique banding patterns from all the others, respectively. In both of these *Rep*-PCR analyses, all of the reference strains formed unique distinctive patterns from the isolates and from each other.

Not only the *Rep*-PCR, but also the ITS fingerprinting profiles of these isolates and strains confirmed their genotypic diversity (Fig. 2, Table III and IV). The

Fig. 2. The cumulative 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 and by ITS-, BOX- and GTG-PCR profiles from isolates and reference strains belonging to genus *Anoxybacillus*. The dendrogram was constructed by using UPGMA, with correlation levels expressed as percentage values of the Dice coefficient. The numbers of clusters obtained from the cumulative analysis were indicated in the right side of the figure. The isolates, having unique distinctive profiles, which displayed similarities less than 16.7% for one to four fingerprintings patterns among these six analyses were indicated with "*".


ITS
GTG
BOX
ALU
HAE
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bp
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100
D463*
E206b*
C161ab*
DSM 16325
DSM 14988
DSM 15939
DSM 17141
D211*
D213*
D214*
D98a*
D404
E184b
E206a
E183
E237*
D594
E272
D487
D503
E243*
C163a
E184aa
E208b
E208a*
A321
A402b*
E123*
D52b*
E331
D232a
D433a
DSM 22626
C245*
D205
A414*
A3210*
D394*
D222b*
D591
D486*
D202a
D242b*
D243
D455*
NCIB 13933
D401a
DSM 15730
NCIB 13971
DSM 2641
F81*
D304
D221a
D203b
D36*
D371a
D376b*
NCIMB13972
D392*
DSM 17075
A351a
A352b
A371
E184ab*
DSM 17127
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Table IV
Contents of the clusters of *Anoxybacillus* isolates and reference strains derived from individual ARDRA profiles and ITS-, BOX- and GTG-PCR fingerprintings

	ARDRA profiles of 16S rRNA gene			ITS-PCR profiles	*Rep*-PCR profiles	
	*Alu*I	*Hae*III	*Taq*I		BOC-PCR	GTG-PCR
1	A3210 = D371a	E206a ≈ **2641**	A3210 ≈ D455	A351a = A371 = A352b	A321 = E272	E206b*
2	E208a*	E206b*	D376b*	D463*	D52b = E184b = E208b	**15939***
3	D455 ≈ E184b	D232a ≈ D433a	E272 ≈ E331	**13972***	A3210 = A402b ≈ F81	**13971***
4	E184ab*	**13972***	D594 = E123	D486*	E123 = E331	**2641***
5	D52b*	C163a ≈ D594	A402b = D214	**2641***	D594 = E237	**17075***
6	**15939** = **17141** = **22626** = **13933** = **13972**	**22626***	C161ab*	**15939***	E243*	D401a*
7	**13971**	E123*	**17127***	C161ab*	**22626***	**16325***
8	**17127**	D304 = D221a	**15939** = **17141**	**17075***	D222b = D394	C161ab*
9	C161ab = **17075**	D242b = D243	**17075***	**16325***	**17141***	**13972***
10	D594 = E123 = E272 = E331	D591 ≈ **13971**	D498a = D487 = D503	E184ab = E208b = E184aa = E184b = E208a	E183 = E206b = E184ab	**14988***
11	A402b*	D404 ≈ D463	**22626** = D404	E183 = E206b	D455 = E184aa	D211 = D213 = D214 D98a
12	A352b = A371	D202a = D203b	D401a = D463 = **13972** = **13971**	A3210 = D594	D371a = D36	D404 ≈ E184b
13	A414*	**15939***	D36*	E243 = E272	E208a*	E206a*
14	E183 = E208b	E272 = E331	**2641** = **14988** = **13933**	D214*	D376b = **13933**	E183 = E237
15	D214 ≈ **14988**	A414 = D371a = D376b = D455 = E243 = D392 = D394	D304 = D221a	D455*	D232a = D242b	D594 = E272
16	D304 = D205 = D232a = D392 = D487 = A351a	A321 = A3210 = A402b = C245 = D211 = D213 = D214	D202a = D203b	E237*	D211*	D487 = D503 = E243
17	C163a = D243 = D433a = D486 = D503 = E206a = E206b = **2641** = D591	D98a*	A414*	E123 = E331	D591*	C163a = E184aa = E208b
18	D401a = D463 = D404 = D98a = D202a	**14988***	D211*	D371a = D52b ≈ 13933	C163a = D213	E208a*
19	D221a = D242b = D203b	D487 ≈ D503	A3210*	D394*	D503*	A321 = A402b = E123
20	A321 ≈ D394	D36*	D243 = D591	**22626***	C245*	D52b = E331
21	D376b*	A352b = D205 = A371 = A351a = E183 = D401a ≈ **17141**	D242b*	D222b*	D98a = D214 = E06a	D455*
22	E237 ≈ E243	E184ab = E208a = E208b = F81 = E184aa = E184b = E237 = D222b = D52b	E243*	F81*	D202a = D205 = D433a	A3210 = D394

Table IV continued

	ARDRA profiles of 16S rRNA gene			ITS-PCR profiles	*Rep*-PCR profiles	
	*Alu*I	*Hae*III	*Taq*I		BOC-PCR	GTG-PCR
23	D22b*	**13933** ≈ **17127**	D486 = E206b = E206a	D487 = D503	A414*	C245 ≈ D205
24	C245 ≈ F81	C161ab = **17075**	D371a = D394	D376b*	D243*	A414*
25	D211 ≈ D213	D486*	C163a = D433a = D232a	C163a ≈ **13971**	D487*	D222b = D591
26	E184aa ≈ D36	**15730***	C245*	D98a ≈ E206a	**13972***	D486*
27	**15730***	**16325***	E184ab = E184b = A371 = D205 = D222b = E183 = E184aa = E208a = D52b = E208b = F81 = E237 = A351a = A352b	D401a = D404	**16325***	D202a = D242b = D243
28	**16325***	–	D392*	**17141***	D221a ≈ **13971**	**22626***
29	–	–	D213*	A414 = C245 = A402b	D203b = D304	**13933***
30	–	–	**15730***	D392 = D36	**15939***	F81*
31	–	–	**16325***	D211 = D213 = A321	**17127***	D232a = D433a = D203b
32	–	–	–	**14988***	D486*	D371a = D376b
33	–	–	–	**17127***	**2641***	D36 = D304 = D221a
34	–	–	–	D205 = D242b = D232a = D202a = D203b = D304 = D221a = D243 = D433a = D591	A352b = A371 = A351a	D392*
35	–	–	–	**15730***	**17075***	**17141***
36	–	–	–	–	D401a = D404	D463*
37	–	–	–	–	C161ab*	A351a = A352b = A371
38	–	–	–	–	**14988***	E184ab*
39	–	–	–	–	D463*	**17127***
40	–	–	–	–	D392*	**15730***
41	–	–	–	–	**15730***	–

The reference strains, indicated with bold characters, were abbreviated from; *A. flavithermus* DSM 2641^T, *A. kestanbolensis* NCIB 13971^T, *A. gonensis* NCIB 13933^T, *A. ayderensis* NCIB 13972^T, *A. thermarum* DSM 17141^T, *A. kamchatkensis* DSM 14988^T, *A. amylolyticus* DSM 15939^T, *A. rupiensis* DSM 17127^T, *A. voinovskiensis* DSM 17075^T, *A. tepidamans* DSM 16325^T, *A. thermarum* DSM 17141^T, *A. salavatliensis* DSM 22626^T. The bacteria having: "≈"; the similar (displaying similarities between 75.0–99.9%), "="; the same (100% similarity), "*"; the unique and distinctive (similarities between 0–16.7%) profiles on the fingerprinting tests used.

ITS-PCR products of the 53 *Anoxybacillus* isolates and 12 type strains generated totally 35 clusters. As in the case of *Rep*-PCR analyses, while the ITS banding patterns of all the type strains showed distinctive profiles from each other and from all the isolates, 10 of the isolates displayed unique fingerprintings from all the others. In addition, none of the isolates shared banding patterns with the reference strains.

In conclusion, it is obvious that the ITS-PCR, $(GTG)_5$-PCR and BOX-PCR products generated a high number of bands giving discriminative information below species and subspecies level between these *Anoxybacillus* isolates and strains studied, when compared with the ARDRA analyses of their 16S rRNA genes. Therefore, as the higher numbers of banding patterns were obtained, and as none of the isolates showed the same profile with the references, the cluster analyses of the *Rep*- and ITS-PCR fingerprintings allowed us to differentiate these isolates and reference strains genetically from each other, and also to group them the in higher numbers according to their distinctive fingerprints.

Discussion

The 16S rRNA gene is known to be a good molecular clock as its primary structure is highly conserved, and thus 16S rRNA gene sequencing is one of the widely used standard techniques in modern bacterial taxonomy by forming the basis of the bacterial phylogeny (Rosello-Mora, 2005). It is also routinely used to apply the rRNA gene technology as a part of 'polyphasic approach' when new descriptions of bacterial species or higher taxa are made (Ludwig and Schleifer, 1999). There are some limitations when comparing the 16S rRNA gene sequences of phylogenetically homogeneous groups of bacteria as in the case of the genus *Anoxybacillus*. One of the limitation factors is that the structurally conserved sequences found in 16S rRNA gene might not allow strains to be identified up to species level in closely related microorganisms (Rodas *et al*., 2003). On the other hand, the Ad Hoc Committee strongly recommended the DNA-DNA hybridizations in cases of species descriptions, when 16S rRNA gene sequences of the novel species show 97.0% or more similarity with its closest relatives (Stackebrandt and Goebel 1994; Stackebrandt *et al.*, 2002; Logan *et al.*, 2009).

The 16S rRNA gene sequence analyses carried out in this study revealed that genus *Anoxybacillus* with its 53 isolates was the most dominant genus having carbohydrate degrading activities in the examined geothermal regions of Turkey. Nearly all of the *Anoxybacillus* isolates were also found to be able to produce carbohydrate degrading enzymes such as amylase and glucosidase. This could be explained by the often occurrence of starch not only in nature and but also in these extreme hot environments and its easy degradation (Derekova *et al.*, 2008). In addition, the presence of biotechnologically important enzyme producing *Anoxybacillus* isolates was also determined for further researches.

Comparison of the generated 16S rRNA gene sequences with all the other described *Anoxybacillus* type strains in the GenBank database indicated that the thermophilic *Anoxybacillus* isolates were mainly diverged into eight phylogenetic groups. The majority of the identified isolates from geothermal areas of Turkey showed the most similarity to their closest relatives; *A. salavatliensis* and *A. kamchatchensis* subsp. *asaccharedens* type strains. Furthermore, based on these phylogenetic analyses, it was determined that all the identified fifty one *Anoxybacillus* isolates were found to be related within the phylogenetically homogenous *Anoxybacillus* type strains of *A. flavithermus*, *A. flavithermus* subsp. *flavithermus*, *A. salavatliensis*, *A. thermarum*, *A. kamchatkensis* and *A. kamchatkensis* subsp. *asaccharedens* which shared 16S rRNA gene sequence similarity values from 97.4% to 99.9%. As this homogenous group of *Anoxybacillus* type strains, demonstrated a significant amount of high intragenic sequence similarity, all of them were closely related species and could only be differentiated from each other by DNA-DNA hybridization analysis, according to the recommendations of the Ad Hoc Committee.

On behalf of these explanations, the identified 51 *Anoxybacillus* isolates, related with these *Anoxybacillus* type strains, showed more than 97.0% sequence similarity (except A321, and C161ab) to more than one closest relatives. Thus, it seems to be that most of the isolates require to be hybridized with their closest relatives as in the case of the reference strains, and it can also be concluded that 16S rRNA sequencing is not generally an informative and conclusive way for the identification of species from this genetically homogeneous members belonging to genus *Anoxybacillus*.

Moreover, isolate A321, which was branched in Cluster 1 with the other isolates resembling *A. salavatliensis*, could not be identified as an *A. salavatliensis* species as it had a sequence similarity value lower than 96.0% to this strain and to all the other described *Anoxybacillus* type strains. As in the case of A321 isolate, C161ab which was the unique member of the Cluster 7 also proposed to be a novel species due to its low level 16S rRNA gene sequence similarities (<97.0%) among all the validly published *Anoxybacillus* type strains. Therefore, this study introduced two potentially novel *Anoxybacillus* species and the sequence comparisons and fingerprinting patterns of these two isolates were promising their novelty among genus *Anoxybacillus* and lead to their further genotypic and phenotypic analysis.

In addition, ARDRA profiles have been well-studied among the thermophilic, spore-forming bacteria, but there is no report demonstrating the ARDRA patterns for the species of the genus *Anoxybacillus* (Caccamo *et al.*, 2001; Kuisiene *et al.*, 2002). Therefore, the restriction patterns of the amplified PCR products digested with *Alu*I, *Hae*III and *Taq*I restriction enzymes of the *Anoxybacillus* isolates and reference strains were taken into cluster analyses. In total 28, 27 and 31 experimental ARDRA profiles were observed, based on the individual *Alu*I, *Hae*III and *Taq*I digestions, respectively. Among these patterns, the *Taq*I restriction sites were higher than the other two restriction enzymes, thus the *Taq*I restriction fragments were thought to be generally more differentiative for genus *Anoxybacillus* with its 31 clusters, whereas only the clusters obtained from *Hae*III-ARDRA analysis were able to differentiate the 12 reference strains to 11 unique clusters. Nevertheless, since the ARDRA analyses were carried out from conserved 16S rRNA gene, the results were not so satisfactory to identify the *Anoxybacillus* isolates and this factor mostly limited distinguishing the *Anoxybacillus* isolates and reference strains up to species level from each other.

The *R*epetitive *E*xtragenic *P*alindromic (*Rep*) elements such as BOX and $(GTG)_5$ are known as evolutionary conserved sequences in prokaryotic genomes and can be used as oligonucleotide binding sites for PCR-mediated genomic fingerprinting (*Rep*-PCR). The *Rep*-PCR can be used to discriminate strains at the species and subspecies level (Versalovic *et al.,* 1994). *Rep*-PCR has been used in a few studies for the determination of the genetic diversity among the *Anoxybacillus* species (De Clerck *et al.,* 2004; Adiguzel *et al.,* 2009; Cihan *et al.,* 2011; Inan *et al.,* 2011). All these studies concluded that the *rep*-PCR fingerprinting technique was a rapid, easy-to-perform, and reproducible tool for differentiation of thermophilic bacteria at the species and subspecies level. Beside these fingerprinting methods, the intergenic transcribed spacers (ITS) between the 16S and the 23S rRNA genetic loci are frequently used in PCR fingerprinting to discriminate at the species and intraspecies levels. The polymorhism in the ITS is due to the presence of tRNA genes that contain multiple ribosomal operons which differ in length and sequence (Daffonchio *et al.*, 2003). This polymorphism was only used for discriminating *A. gonensis* and *A. salavatliensis* from their closely related *Anoxybacillus* species in previous studies of Belduz *et al.* (2003) and Cihan *et al.* (2011).

In the individual cluster analyses of all the *Anoxybacillus* isolates and type strains derived from the $(GTG)_5$ and BOX-PCR, and ITS-PCR fingerprintings, totally 41, 40, and 35 clusters were formed. Furthermore, all these three tests allowed us to differentiate the 12 *Anoxybacillus* reference strains not only from each other, but also from all the isolates. As these tests were determined on non-conservative 16S-23S rRNA gene sequences like ITS region, or on repetitive genetic elements throughout the whole genome such as $(GTG)_5$ and BOX elements, the results were more informative about the genetic diversity of the isolates and reference strains. None of the isolates represented the same banding patterns with the reference strains in the *Rep*- and ITS-PCR analyses, which were formerly found as sharing high 16S rRNA gene sequence similarities and therefore they may not be assigned to their closely related species according to these results.

In conclusion, when these six fingerprintings were combined in a cumulative cluster analysis, a total of 34 clusters was obtained (Fig. 2). In addition, 35 of 53 isolates showed unique patterns different from all the other isolates and reference strains at least from two of these six fingerprinting analyses used. The ITS-PCR, $(GTG)_5$-PCR and BOX-PCR products generated a high number of bands giving discriminative information below species and subspecies level between these *Anoxybacillus* isolates and references, when compared with the ARDRA analyses. The *Rep*- and ITS-PCR fingerprinting patterns allowed us to differentiate these isolates and reference strains genetically, and also to group them in higher numbers according to their distinctive fingerprints. Therefore, we could certainly conclude that most of our isolates genetically diverged from the reference strains at least from the species or subspecies level.

This research also revealed that the ability of carbohydrate degradation effects the high population of genus *Anoxybacillus* in these geothermal regions and make them superior among the other thermophilic endospore-forming bacilli in these habitats. In this study, not only the phylogenetic diversity of a large collection of isolates belonging to genus *Anoxybacillus* were analyzed, but also the efficiency of 16S rRNA gene sequencing, ARDRA techniques, *rep*-PCR and also ITS-PCR fingerprintings were discussed. According to this survey, the genus *Anoxybacillus* was found to be genetically a very homogenous group of bacteria as both isolates and type strains showed high levels of intragenic sequence 16S rRNA gene similarities and isolates having more than 97% sequence similarities need to be DNA-DNA hybridization analysis with their closest relatives. Thus, most of the isolates will have to be hybridized with more than one relative. Nevertheless, when these easy, having low-cost and single-performance fingerprinting techniques of *Rep*- and ITS-PCR were applied before carrying out high-cost and labour-requiring hybridization tests with all the closest relatives, they would be very helpful to decide which relatives need to be hybridized according to the differences in their fingerprinting patterns. This will also aid to decrease the number of hybridization analyses with some of these relatives, which will give low similarities than expected, as revealed before in the first identification and description of type strain *A. salavatliensis* (Cihan *et al*., 2011).

Although there were studies with these fingerprinting techniques, this is the first, which a large number of *Anoxybacillus* isolates and reference strains were used by a polyphasic approach and which the success of these tests were demonstrated on characterization and differentiating *Anoxybacillus* isolates. All these results concluded that especially when *Rep*-PCR and ITS-PCR fingerprinting techniques were combined with other 16S rRNA gene sequence based analyses, they would consider as easy and accurate genotypic tools before deciding the hybridization pairs for the identification purposes.

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