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Use of *rpoB* Sequences and rep-PCR for Phylogenetic Study of *Anoxybacillus* Species

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This study was conducted to investigate the applicability of *rpoB*, which encodes the β subunit of RNA polymerase, to be used as an alternative to 16S rRNA gene sequence similarity analysis in the thermophilic genus *Anoxybacillus*. Partial *rpoB* sequences were generated for the 14 type strains of *Anoxybacillus* species and 6 other strains of four *Anoxybacillus* species. The sequences and the phylogenetic tree of *rpoB* were compared with those obtained from 16S rRNA gene analysis. The *rpoB* gene was found to provide a better resolution for *Anoxybacillus* species, with lower interspecies sequence similarities. The *rpoB* sequence similarity analysis permitted a more accurate discrimination of the species within the *Anoxybacillus* genus than the more commonly used 16S rRNA gene. Furthermore, rapid and reproducible repetitive extragenic palindromic fingerprinting techniques (REP-, ERIC-, and BOX-PCR) were employed for the specimens of genus *Anoxybacillus*. Through comparison of the three methods, it was found that the BOX-PCR method generated more informative results than REP-PCR for the studied strains; BOX-PCR profiles were more distinct for the different strains, including a higher number of bands. Rapid and reproducible repetitive extragenic palindromic fingerprinting techniques (rep-PCR) constitute a suitable molecular approach for the validation and maintenance of taxonomy within the *Anoxybacillus* genus. The results of this study show that *rpoB* and rep-PCR provide rapid and reliable methods for molecular typing of *Anoxybacillus* species.

***Keywords*:** *Anoxybacillus*, 16S rRNA, *rpoB*, REP-PCR, BOX-PCR, ERIC-PCR

The genus *Anoxybacillus* was separated from the genus *Bacillus*, with the type species as *Anoxybacillus pushchinoensis* (Pikuta *et al*., 2000). The genus *Anoxybacillus* encompasses aerotolerant anaerobes, aerobes, or facultative anaerobes, alkaliphilic or alkalitolerant, moderately thermophilic, endospore-forming bacteria. *Anoxybacillus* species are widely distributed and readily isolated from geothermally heated environments. There is an increasing industrial interest in their thermostable gene products. Therefore, studying the phylogenetic relations and diversity in this interesting bacterial genus is not only a taxonomical concern, but also a useful route towards full development of its biotechnological potential.

The *ad hoc* committee for the re-evaluation of the species definition in bacteriology (Stackebrandt *et al*., 2002) recommended evaluation of protein-coding gene sequence analysis for its applicability to genomically circumscribe the taxon species and differentiate it from neighboring species detected by, for example, rRNA gene sequences. Protein-coding genes have advantages over ribosomal genes as molecular markers in that they offer a large number of unlinked sources of phylogenetic information (Tessmann *et al*., 2001). In addition, the amino acid sequences of protein-coding genes can often resolve some of the deeper branches in a tree, except for the wobble bias on the third nucleotide of a codon sequence. In spite of the disadvantage of the wobble bias within a protein coding gene, one possibility is to use the protein-coding genes, because the degeneracy of the genetic code indicates that even when a protein is completely conserved, the DNA sequence encoding that protein can vary (Barton *et al*., 2007).

The gene for the RNA polymerase beta subunit, *rpoB*, has been introduced in taxonomic studies and community analyses of bacteria as an alternative to the 16S rRNA gene, because it exists in a single copy in all bacterial genomes studied so far, and contains conserved as well as variable regions (Da Mota *et al*., 2004). Analysis of the *rpoB* gene encoding the beta-subunit of RNA polymerase has been used successfully as a genotyping approach to overcome obstacles from 16S rRNA gene similarity in phylogenetic studies within *Geobacillus*, *Paenibacillus*, *Staphylococcus*, *Bacillus*, *Mycobacterium*, *Mycoplasma*, *Complobacter*, *Pasteurellaceae*, and *Enterobacteriaceae* (Mollet *et al*., 1997; Peixoto *et al*., 2002; Drancourt and Raoult, 2002; Kim *et al*., 2003; Ko *et al*., 2003; Da Mota *et al*., 2004; Korczak *et al*., 2004, 2006; Meintanis *et al*., 2008; Weng *et al*., 2009). However, the value of the *rpoB* sequence as a basis for species identification has not yet been assessed for *Anoxybacillus*.

Repetitive-sequence-based polymerase chain reaction (rep-PCR), a genomic fingerprinting technique, has been described as a powerful molecular typing method to determine taxonomic and phylogenetic relationships among bacteria. The rep-PCR technique is simple, can differentiate between closely related strains of bacteria, and can assign the bacteria potentially up to the strain level based on the presence of repeated elements within the genome examined (Rameshkumar and Nair, 2009). However, to date, no genomic fingerprinting techniques have

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Table 1. GenBank accession nos. of partial *rpoB* and 16S rRNA gene sequences for representative strains of *Anoxybacillus* species investigated in this study

Species	Strain number	GenBank Accession Number	
		16S rRNA	*rpoB*
Anoxybacillus amylolyticus	* DSM 15939^T (Poli *et al*., 2006)	AJ618979	JF279467
Anoxybacillus ayderensis	‡ NCIMB 13972^T (Dulger *et al*., 2004)	AF001963	JF279476
Anoxybacillus bogrovensis	* DSM 17956^T (Atanassova *et al*., 2008)	AM409184	JF279468
Anoxybacillus contaminans	* DSM 15866^T (De Clerck *et al*., 2004)	AJ551330	JF279478
Anoxybacillus eryuanensis	‡ KCTC 13720^T (Zhang *et al*., 2011)	GQ153549	JF279469
Anoxybacillus flavithermus	* DSM 2641^T (Pikuta *et al.*, 2000)	Z26932	JF279475
Anoxybacillus flavithermus	† WK1 (Saw *et al*., 2008)	CP000922†	CP000922†
Anoxybacillus gonensis	‡ NCIMB 13933^T (Belduz *et al.*, 2003)	AY122325	JF279473
Anoxybacillus gonensis	‡ A4 (Belduz *et al*., 2003)	AY248707	JF279485
Anoxybacillus gonensis	‡ A7 (Belduz *et al*., 2003)	AY248708	JF279481
Anoxybacillus gonensis	‡ I3 (KTU, Turkey)	GQ265907	JF279484
Anoxybacillus kamchatkensis	* DSM 14988^T (Kevbrin *et al*., 2005)	AF510985	JF279479
Anoxybacillus kestanbolensis	‡ NCIMB 13971^T (Dulger *et al*., 2004)	AY248711	JF279474
Anoxybacillus pushchinoensis	* DSM 12423^T (Pikuta *et al*., 2000)	AJ010478	JF279477
Anoxybacillus pushchinoensis	‡ *A8* (Kacagan *et al*., 2008)	AY248715	JF279482
Anoxybacillus rupiensis	* DSM17127^T (Derekova *et al*., 2007)	AJ879076	JF279471
Anoxybacillus tenghongensis	‡ KCTC 13721^T (Zhang *et al*., 2011)	FJ438370	JF279470
Anoxybacillus thermarum	* DSM 17141^T (Poli *et al*., 2009)	JF292514#	JF279472
Anoxybacillus voinovskiensis	§ TH13 (Yumoto *et al*., 2004)	AB110008	JF279483
Anoxybacillus voinovskiensis	‡ I4.2 (KTU, Turkey)	GQ265906	JF279480

‡ The strain was isolated by us and defined according to their DNA-DNA relatedness
* The *Anoxybacillus* type strain purchased from DSM; Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures)
‡ The *Anoxybacillus* type strain purchased from KCTC; Korean Collection for Type Cultures
§ The strain was kindly provided by Isao Yumoto at National Institute of Advanced Industrial Science and Technology, Sapporo, Japan.
† We were retrieved 16S rRNA and *rpoB* sequences from completed genome in GenBank
# The 16S rRNA sequence identified in this study and deposited in GenBank.

been applied to the taxonomic study of *Anoxybacillus* species.

In this study, the phylogenetic relationships of 14 type strains of genus *Anoxybacillus* and 6 other strains representing 4 *Anoxybacillus* species (*A. gonensis*, *A. voinovskiensis*, *A. pushinoensis*, *A. flavithermus*) were investigated by means of rep-PCR, *rpoB*, and 16S rRNA gene sequence, and were demonstrated in phylogenetic trees constructed with 16S rRNA gene and *rpoB* sequences. The effectiveness of rep-PCR and *rpoB* sequence for differentiating and grouping strains belonging to the genus *Anoxybacillus* is discussed. This is the first report on the application of genotypic methods (rep-PCR and *rpoB* gene) as taxonomic analysis tools in *Anoxybacillus.*

Materials and Methods

Bacterial strains and general methods

The type strains and other strains of *Anoxybacillus* used in this study and their sources are listed in Table 1. All the strains were streaked on Luria Bertani agar (LBA) plates and cultured at 50°C for 16 h; purity was confirmed by macroscopic examination of the colonies. Chromosomal DNA was extracted from all strains of *Anoxybacillus* using the Wizard Genomic DNA Purification kit (Promega Corporation, USA).

PCR amplification and DNA sequencing

To generate sequences longer than those available in GenBank, the 16S rRNA gene of *A. thermarum* DSM 17141^T was selectively amplified from purified genomic DNA by using oligonucleotide primers designed to anneal the conserved positions in the 5′ and 3′ regions of bacterial 16S rRNA genes. The forward primer, UNI16S-L (5′-ATTCTAGAGTTTGATCATGGCTTCA-3′), corresponded to positions 11-26 of *Escherichia coli* 16S rRNA, and reverse primer, UNI16S-R (5′-ATGGTACCGTGTGACGGGCGGTGTTGTA-3′), corresponded to the complement of positions 1411-1393 of *E. coli* 16S rRNA (Brosius *et al*., 1978). The PCR reaction conditions were made according to Beffa *et al*. (1996).

For amplification of *Anoxybacillus rpoB* gene sequences, the *rpoB* primers used were rpoB1698F (5′-AACATCGGTTTGATCAAC-3′, corresponding to *E. coli* position 1643) and rpoB2041R (5′-CGTTGCATGTTGGTACCCAT-3′, corresponding to *E. coli* position 2041) (Dahllöf *et al*., 2000). The reaction mix contained 1 μl of template DNA (50-100 ng), 10 mM of Tris-HCl, pH 8.3, 10 mM of KCl, 25 pmol of each primer, 2.5 mM of each deoxynucleoside triphosphate, 20 μg of BSA, 2.6 mM of $MgCl_2$, and 5 U/50 μl of Taq polymerase. The PCR protocol consisted of a denaturing step of 94°C for 5 min, followed by 25 cycles of denaturing for 30 sec at 94°C, annealing for 1.5 min at 50°C, and a 1.5-min extension at 72°C. Subsequently, a final extension step of 72°C for 10 min was performed. The PCR products were run on a 1.4% agarose gel containing ethidium bromide, and analyzed using the Bio-Rad Gel Doc imaging system.

16S rRNA and *rpoB* PCR products were cloned using the pGEM T-easy vector, according to the instructions of the manufacturer (Promega). 16S rRNA and *rpoB* gene sequences were determined with an Applied Biosystems model 373A DNA sequencer by using the

Table 2. Strains of *Anoxybacillus* used in this study and percent similarity among the 16S rRNA and *rpoB* gene sequences of the *Anoxybacillus* species

	rpoB																			
	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *A. amylolyticus* DSM 15939^T		**80**	**77**	**83**	**82**	**79**	**78**	**79**	**79**	**79**	**78**	**80**	**80**	**80**	**81**	**81**	**80**	**79**	**83**	**80**
2. *A. ayderensis* NCIMB 13972^T	96		**79**	**82**	**89**	**90**	**89**	**97**	**97**	**96**	**97**	**96**	**91**	**92**	**92**	**79**	**79**	**78**	**89**	**95**
3. *A. bogrovensis* DSM 17956^T	95	96		**78**	**79**	**79**	**79**	**79**	**78**	**78**	**79**	**77**	**79**	**78**	**79**	**75**	**76**	**76**	**79**	**77**
4. *A. contaminans* DSM 15866^T	98	97	94		**81**	**84**	**83**	**82**	**81**	**81**	**81**	**84**	**84**	**84**	**84**	**82**	**83**	**81**	**81**	**81**
5. *A. eryuanensis* KCTC 13720^T	96	97	96	97		**90**	**91**	**89**	**89**	**89**	**88**	**88**	**91**	**89**	**78**	**79**	**78**	**89**	**96**	**88**
6. *A. flavithermus* DSM 2641^T	96	98	96	97	99		**98**	**89**	**89**	**89**	**89**	**88**	**96**	**89**	**88**	**78**	**80**	**80**	**91**	**89**
7. *A. flavithermus* WK†	96	98	97	97	99	99		**88**	**88**	**88**	**88**	**87**	**95**	**87**	**87**	**76**	**79**	**79**	**92**	**88**
8. *A. gonensis* NCIMB 13933^T	96	98	96	96	97	98	98		**99**	**99**	**99**	**95**	**90**	**91**	**91**	**78**	**78**	**78**	**89**	**94**
9. *A. gonensis* A4	96	99	96	97	98	98	98	99		**99**	**99**	**95**	**90**	**91**	**91**	**78**	**78**	**78**	**89**	**93**
10. *A. gonensis* A7	96	98	96	96	97	98	98	98	99		**99**	**94**	**90**	**90**	**90**	**78**	**78**	**78**	**89**	**93**
11. *A. gonensis* I3	96	98	96	97	97	98	98	98	99	98		**95**	**90**	**91**	**90**	**77**	**78**	**78**	**89**	**93**
12. *A. kamchatkensis* DSM 14988^T	96	99	96	97	97	98	98	98	99	99	99		**89**	**90**	**90**	**78**	**80**	**80**	**88**	**95**
13. *A. kestanbolensis* NCIMB 13971^T	95	97	96	96	97	98	98	97	97	97	97	97		**89**	**88**	**79**	**80**	**90**	**92**	**90**
14. *A. pushchinoensis* DSM 12423^T	95	97	96	96	98	98	98	97	97	97	97	97	97		**98**	**79**	**77**	**77**	**89**	**88**
15. *A. pushinoensis* A8	95	97	96	96	98	98	98	97	97	97	97	97	98	98		**79**	**78**	**78**	**88**	**88**
16. *A. rupiensis* DSM 17127^T	97	95	95	96	95	95	95	95	95	95	95	95	94	94	94		**79**	**78**	**79**	**76**
17. *A. voinovskiensis* NCIMB 13956^T	98	95	94	98	95	95	95	95	95	95	95	95	94	94	94	96		**98**	**78**	**79**
18. *A. voinovskiensis* I4.2	98	96	95	98	96	96	96	95	96	96	96	96	95	95	95	96	98		**78**	**79**
19. *A .tenghongensis* KCTC 13721^T	96	97	96	96	98	98	98	97	97	97	97	97	97	98	98	95	95	95		**87**
20. *A. thermarum* DSM 17141^T	97	99	96	97	97	98	99	98	99	99	99	99	97	98	97	95	95	96	97	
	16S rRNA																			

* The numbers across the top correspond to the species on the left; ^{T} type strain.

ABI PRISM cycle sequencing kit (Macrogen, Korea). The sequence of 16S rRNA gene of *A. thermarum* DSM 17141^T and the sequences of the *rpoB* genes of *Anoxybacillus* type strains and strains, as depicted in Table 1, have been deposited in the GenBank database.

Phylogenetic analysis

The 16S rRNA gene of *A. thermarum* DSM 17141^T was determined in this study to obtain longer sequence fragments. The 16S rRNA gene sequences of the other 13 type strains of *Anoxybacillus* and 6 other strains representing 4 species of *Anoxybacillus* were retrieved from the GenBank database.

The *rpoB* sequences of 14 type strains and 5 other strains of *Anoxybacillus* were obtained in this study. The *rpoB* sequence of *A. flavithermus* WK1 was retrieved from the completed genome from GenBank (Accession number, CP000922).

16S rRNA and *rpoB* genes were aligned using the multiple alignment program, CLUSTAL X (Thompson *et al*., 1997). Evolutionary distances were calculated by using Kimura's two-parameter model (Kimura, 1980). Phylogenetic analyses were performed by the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis based on 1,000 replicates was also conducted to obtain confidence levels for the branches (Felsenstein, 1985). The phylogenetic trees were constructed using the program, MEGA4 (Tamura *et al*., 2007).

Genomic fingerprinting

A total of 14 type strains and 5 other strains were subjected to rep-PCR genomic fingerprinting using primer sets corresponding to REP, ERIC, and BOX elements (Versalovic *et al*., 1991, 1994; Gevers *et al*., 2001). The 18-mer primer pair REP1R (5′-IIIICGICGICATCIG GC-3′) and REP2I (5′-ICGICTTATCIGGCCTAC-3′) (where I is Inosine), ERIC 1R (5′-ATGTAAGCTCCTGGGGAT-3′) and ERIC 2 (5′-AAGTAAGTGACTGGGGGTGAGC-3′), and BOX A1R (5′-CTA CGGCAAGGCGACGCTGACG-3′), were used to amplify putative REP-, ERIC-, and BOX- like elements in the bacterial DNA, respectively. The PCR and electrophoresis conditions were carried out according to Adiguzel *et al*. (2009).

Results

Phylogenetic analysis of 16S rRNA gene sequences

The relationships among *Anoxybacillus* species were analyzed according to the similarities in their 16S rRNA gene sequences. The sequences of 16S rRNA gene in the representative *Anoxybacillus* strains from GenBank databases and from this study were aligned, and a phylogram was constructed based on 1280 unambiguously aligned positions in the 16S rRNA gene (positions 80-1357, numbered on the basis of *Bacillus subtilis* 16S rRNA gene sequence; GenBank accession number AB042061). The 16S rRNA gene sequence similarity matrix tabulated after CLUSTAL X alignment of these partial 16S rRNA sequences showed that the percent similarity ranged from 94 to 99% (Table 2). The 16S rRNA gene phylogram inferred from the neighbor-joining method is shown in Fig. 1.

rpoB Gene sequencing and phylogenetic analysis

The consensus primer set rpoB1698f/rpoB2041R was used to amplify the partial *rpoB* gene of the 14 type strains and 5 other strains of *Anoxybacillus*. A 359-bp region of the *rpoB* genes (positions 1571-1929, numbered on the basis of *B. subtilis rpoB* gene sequence; GenBank accession number Z99104) was used for phylogenetic analysis. The *rpoB* gene sequences obtained showed 75-99% similarity, permitting a more accu-

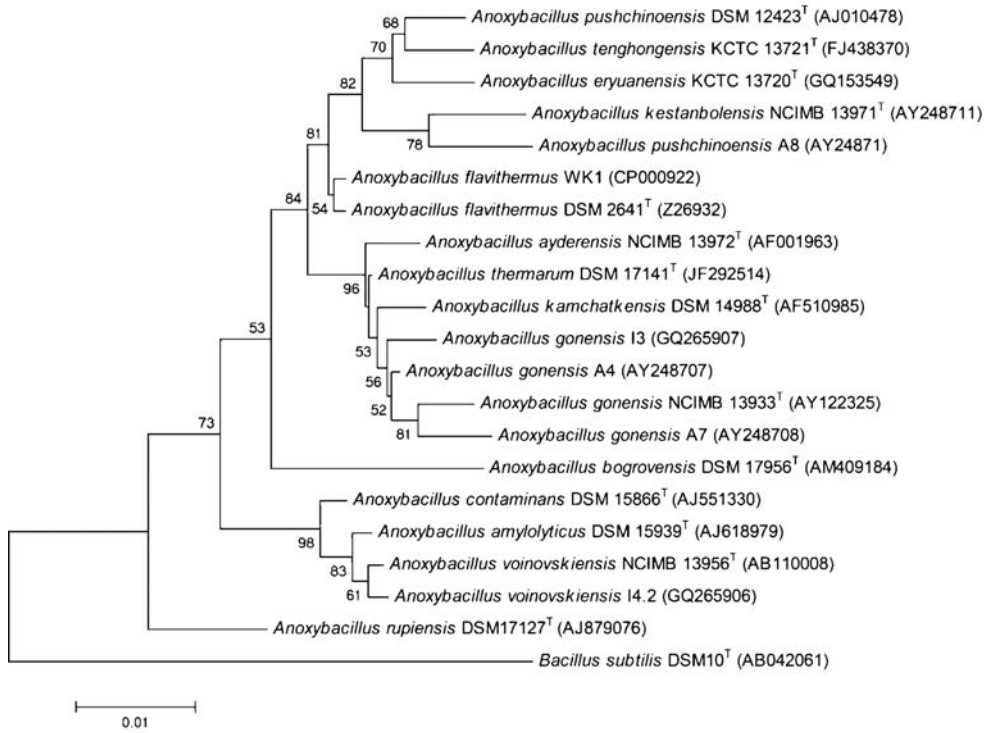

Fig. 1. Neighbour-joining phylogenetic tree of the genus *Anoxybacillus* based on 16S rRNA gene sequences. The species names, strain numbers and accession numbers are given. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at the branch points. *B. subtilis* DSM 10^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

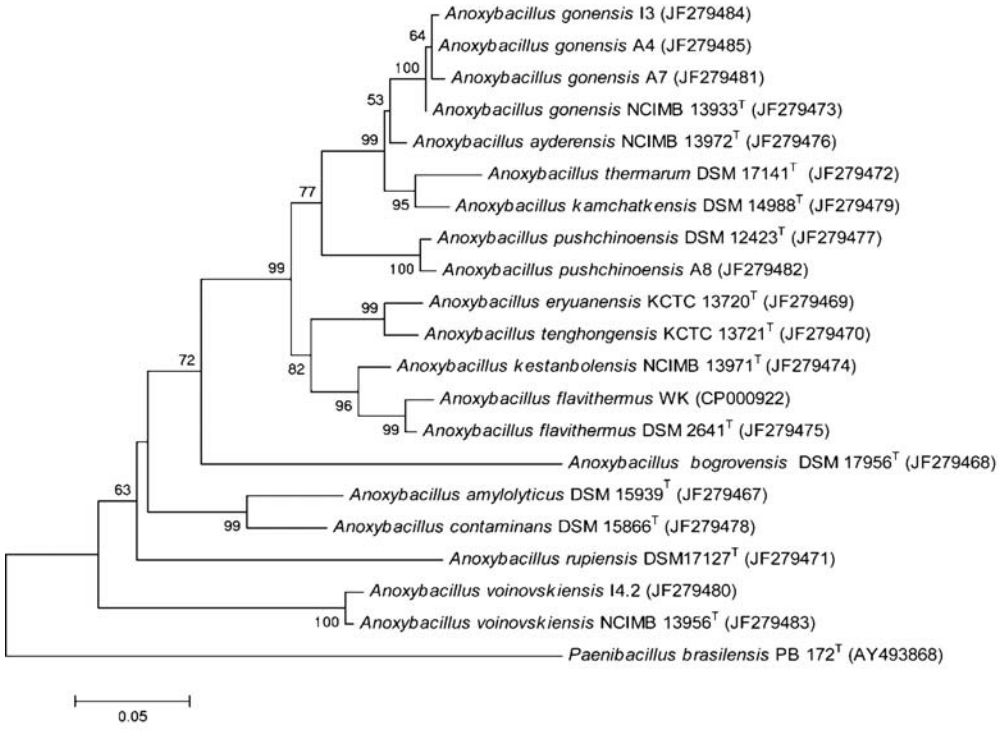

Fig. 2. Neighbour-joining phylogenetic tree of the genus *Anoxybacillus* based on partial *rpoB* sequences. The species names, strain numbers and accession numbers are given. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at the branch points. *P. brasilensis* PB 172^T was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

rate discrimination of the species (Table 2). The *rpoB* phylogram inferred from the neighbor-joining method is shown in Fig. 2.

Comparative sequence analysis of the *rpoB* revealed that *A. gonensis* I3, *A. gonensis* A4, and *A. gonensis* A7 showed ≥98% similarity with *A. gonensis* NCIMB 13933^T; but showed ≤97% similarity with the other species of *Anoxybacillus*. Also, *A. pushchinoensis* A8 and *A. voinovskiensis* I4.2 were highly

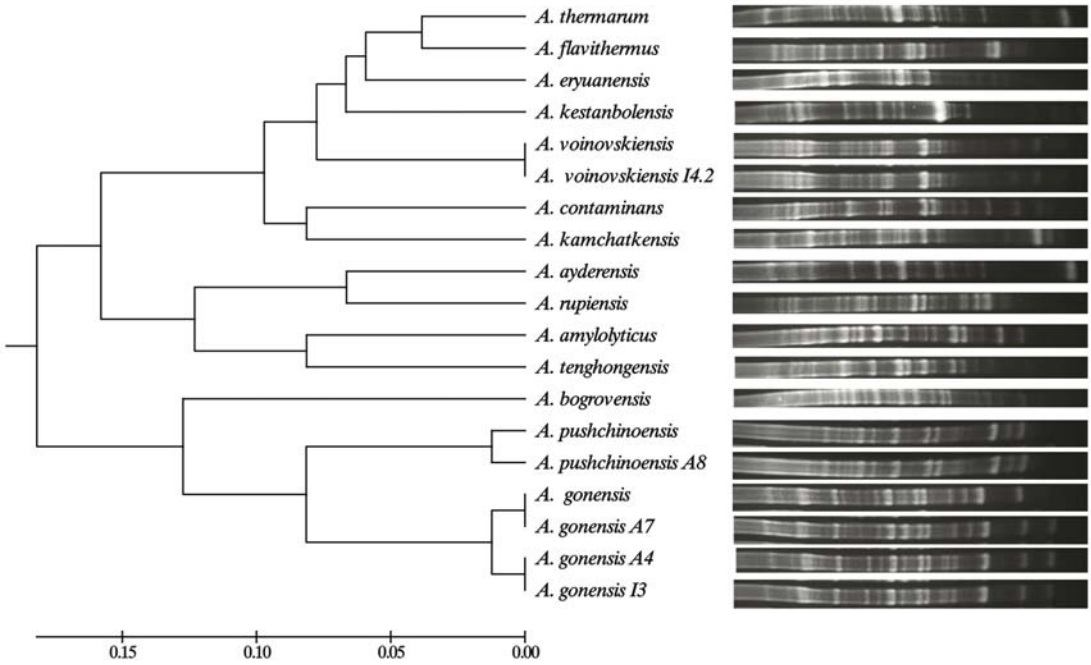

Fig. 3. Cluster analysis of digitized banding patterns, generated by BOX-PCR. The dendrogram was constructed by using UPGMA.

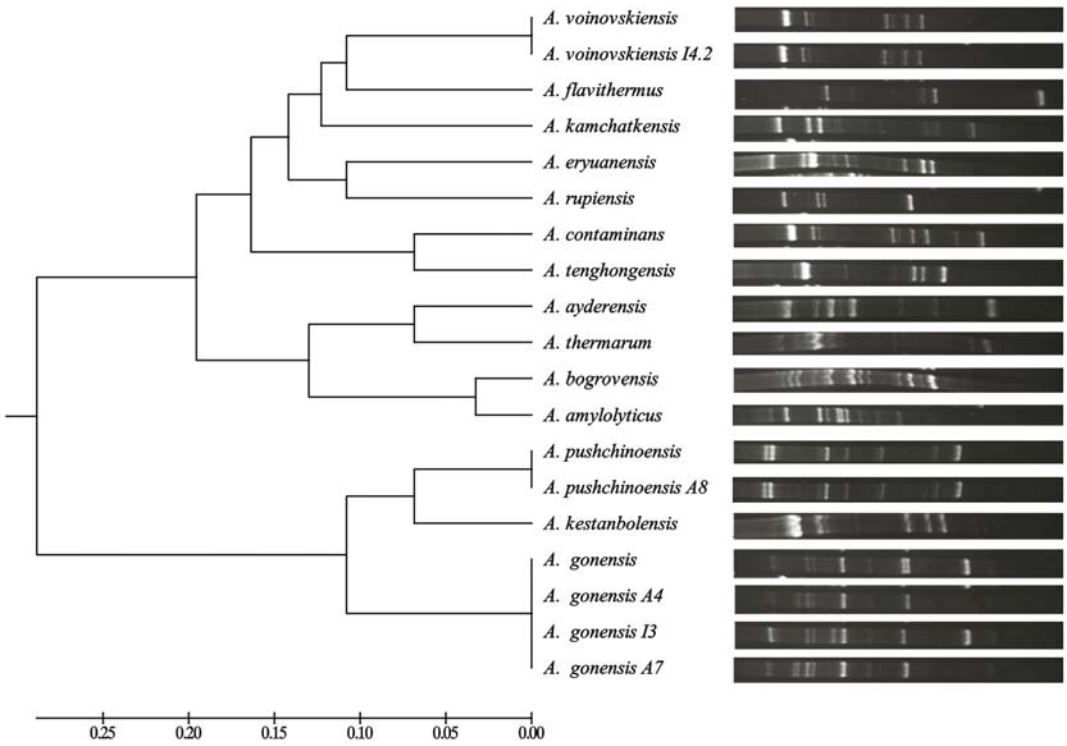

Fig. 4. Cluster analysis of digitized banding patterns, generated by REP-PCR. The dendrogram was constructed by using UPGMA.

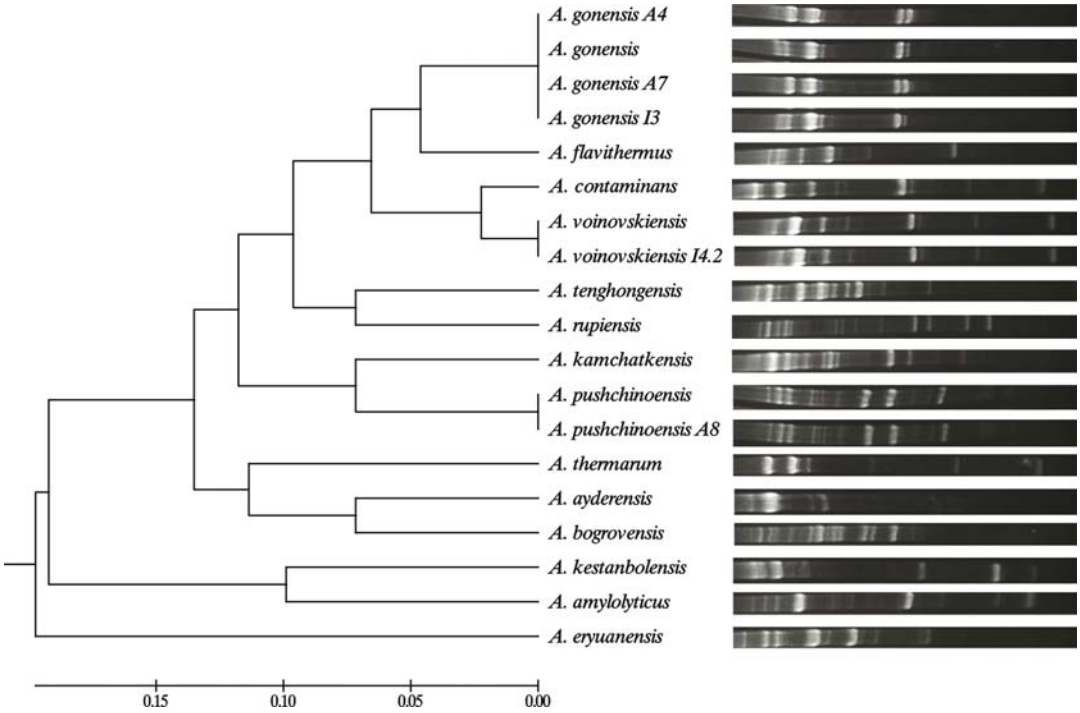

Fig. 5. Cluster analysis of digitized banding patterns, generated by ERIC-PCR. The dendrogram was constructed by using UPGMA.

related to their type strains (*A. pushchinoensis* DSM12423^T and *A. voinovskiensis* NCIMB 13956^T, respectively) with 98% similarity, while they showed <97% similarity with the other species of *Anoxybacillus*. In addition, it was observed that *rpoB* sequences of *Anoxybacillus* type strains showed ≤97% similarity with each other. Thus, this study demonstrated that *rpoB* sequence of the genus *Anoxybacillus* is sufficiently different among the species (75-97% sequence similarity), and sufficiently conserved within the species (≥98% sequence similarity), to be able to identify *Anoxybacillus* species by sequence comparison of *rpoB*. The *rpoB* gene was found to provide a better resolution for *Anoxybacillus* species, with lower interspecies sequence similarities, ranging from 75 to 97%, when compared with those for the 16S rRNA gene (94-99%). Thus, *Anoxybacillus* species can be better differentiated by using the *rpoB* gene than the 16S rRNA gene.

In particular, 16S rRNA and *rpoB* sequence comparisons of type strains of *Anoxybacillus* were performed, confirming >95% similarity for 76.3% of the 16S rRNA gene sequences compared, while the percentage of *rpoB* sequences with >95% similarity was only 3.9%. Furthermore, the percentage of sequences with identity >93% was 100% for 16S rRNA gene and 6.9% for *rpoB*.

rep-PCR genomic fingerprinting

All strains available were successfully discriminated with rep-PCR. In this study, one single oligonucleotide primer, BOXA1R, and two oligonucleotide primer pairs, REP1R-I/REP2-I and ERIC1R/ERIC2, were tested for their ability to type a subset of 14 type strains and 5 other strains that had already been identified based on DNA-DNA hybridization. The 14 type strains produced different banding patterns, while strains within the same species produced the same BOX-, REP-, and ERIC-PCR profiles as their type strains (Figs. 3-5).

A. gonensis I3, *A. gonensis* A4, and *A. gonensis* A7 exhibited only slight differences in BOX-, REP-, ERIC-PCR banding patterns in terms of the presence or absence of bands. Also, these strains produced the same BOX-, REP-, and ERIC-PCR banding patterns with *A. gonensis* NCIMB 13933^T. In addition, *A. pushchinoensis* A8 and *A. voinovskiensis* I4.2 produced the same BOX-, REP-, ERIC-PCR banding patterns as *A. pushchinoensis* DSM12423^T and *A. voinovskiensis* NCIMB 13956^T, respectively.

The BOX-PCR genomic fingerprints showed that bacterial strains have distinct patterns, with 13-17 fragments in the range 7000-250 bp, and with frequently observed faint bands (Fig. 3). The ERIC primer set generated reproducible and differentiating fingerprints, including 6-13 fragments of 7000-500 bp (Fig. 4). The use of REP primers resulted in a banding pattern containing approximately 7-15 visualized PCR products that ranged from 10,000 to 500 bp (Fig. 5).

Discussion

Systematic bacterial studies have been based on 16S rRNA gene-sequence phylogeny (Ludwing and Klenk, 2001). Despite the general use of the 16S rRNA gene as the framework for modern bacterial classification, it often shows limited variation for discrimination of closely related species, such as the *Anoxybacillus* species. Thus, time-consuming and labor-intensive DNA-DNA hybridization remained as the method of choice for identifying *Anoxybacillus*. Rapid, reliable techniques, such as protein-coding gene analysis and rep-PCR techniques were needed for studying the relationships among the

published and recognized species of *Anoxybacillus*.

In this study, the phylogenetic divergence within the thermophilic genus, *Anoxybacillus,* was investigated. Rapid and reliable molecular methods that have previously been applied for identification and classification in a variety of bacterial genera, such as rep-PCR and *rpoB* gene sequence analysis (Meintanis *et al*., 2008), were employed for the specimens of the *Anoxybacillus* genus for the first time in this study.

Analysis of *rpoB* revealed that comparison of partial sequences of *rpoB* for the type strains of *Anoxybacillus* was more sensitive than a 16S rRNA gene comparison, and provided a better resolution for *Anoxybacillus* species. The 14 type strains under investigation showed 94-99% similarity among the 16S rRNA gene sequences analyzed, showing high similarity within the genus *Anoxybacillus.* However, 75-97% similarity was observed among the *rpoB* sequences analyzed, allowing a more accurate discrimination of the species. When the partial *rpoB* sequences (359 bp) were compared with the 16S rRNA (1,280 bp) gene sequences in *Anoxybacillus* to determine the discriminative power of each gene, *rpoB* was found to be about 2.2 times more discriminative than the 16S rRNA gene. Da Mota *et al*. (2004) obtained similar results, as their comparison of the discriminative power of the two genes (16S rRNA and *rpoB*) in the *Paenibacillus* group proved that *rpoB* was 3.1 times more discriminative than 16S rRNA gene.

Furthermore, to confirm the conservation of *rpoB* within the species, we compared the sequences of the *rpoB* derived from 6 strains representing 4 *Anoxybacillus* species (*A. pushchinoensis*, *A. flavithermus*, *A. gonensis*, and *A. voinovskiensis*), which had been identified by DNA-DNA hybridization. After comparing the *rpoB* gene fragments, every strain was identified accurately at the species level, although differences were observed between the sequences of strains of the same species, resulting in an intraspecies similarity, ranging from 98 to 100% (Table 2). The 14 type strains of *Anoxybacillus* showed 75-97% similarity among the *rpoB* sequences analyzed (Table 2). Based on these results, we can draw a threshold value for the *rpoB* gene sequences analyzed in this study at 0-2% sequence divergences among the strains in the same species of *Anoxybacillus.*

The *rpoB* gene sequence had greater resolution than the 16S rRNA gene sequence for the genus *Anoxybacillus*. Comparison of the 16S rRNA genes proved to be insufficient for resolving the phylogenetic relationships between closely related species in *Anoxybacillus.* However, the *rpoB* resolution was sufficient for the differentiation of very closely related species of *Anoxybacillus*, such as *A. thermarum* DSM 17141^T and *A. kamchatkensis* DSM 14988^T; *A. ayderensis* NCIMB13972^T and *A. kamchatkensis* DSM 14988^T; and *A.eryuanensis* KCTC 13720^T and *A. flavithermus* DSM 2641^T, which showed sequence similarity of >99% for 16S rRNA gene. However, the sequence similarities of these species for the *rpoB* gene were 95, 96, and 90% for each pair, respectively.

Only four comparisons of *rpoB* gene sequences showed >95% similarity; *A. gonensis* NCIMB13933^T and *A. ayderensis* NCIMB13972^T; *A. kestanbolensis* NCIMB13971^T and *A. flavithermus* DSM 2641^T; *A. ayderensis* NCIMB13972^T and *A. kamchatkensis* DSM 14988^T; and *A. eryuanensis* KCTC 13720^T and *A. tenghongensis* KCTC 13721^T. These showed sequence similarities of 97, 96, 96, and 96%, respectively, for each of the pairs. The value of DNA-DNA hybridization for each of the pairs was retrieved from the literature, and DNA-DNA similarities were 68.6% for *A.gonensis* NCIMB13933^T and *A. ayderensis* NCIMB13972^T (Dulger *et al*., 2004); 60.4% for *A. kestanbolensis* NCIMB13971^T and *A. flavithermus* DSM 2641^T (Dulger *et al*., 2004); 51% for *A. ayderensis* NCIMB13972^T and *A. kamchatkensis* DSM 14988^T (Kevbrin *et al*., 2005); and 46.4% for *A. eryuanensis* KCTC 13720^T and *A. tenghongensis* KCTC 13721^T (Zhang *et al*., 2011). The species of *Anoxybacillus* that showed high similarity (96-97%) for *rpoB* gene sequence were closely related species of genus *Anoxybacillus* based on DNA-DNA similarity (>46%). Thus, the *rpoB* gene sequence showed some genetic relationship, in agreement with DNA-DNA hybridization results, and the *rpoB* gene sequence reflects DNA-DNA hybridization results better than the 16S rRNA gene.

Similarities of protein sequences deduced from the partial *rpoB* gene in the 20 *Anoxybacillus* strains were examined. As reported previously in an analysis of the *rpoB* gene of members of the genus *Mycobacterium*, *Streptomyces*, and *Kitasatospora* (Kim *et al*., 1999, 2004), DNA sequence variations in the *rpoB* gene among different species are generally concentrated on the third nucleotide of a codon sequence. Therefore, amino acid sequence similarities deduced from the *rpoB* gene between any given pair of strains within the genus *Anoxybacillus* is always higher than that of the corresponding DNA sequence. In the genus *Anoxybacillus*, the amino acid sequence similarity at the interspecies level ranged from 85 to 100%, whereas, the DNA sequence similarity at the interspecies level ranged from 75 to 97%. Therefore, as determined by Kim *et al.* (2004), the deduced amino acid sequences from *rpoB* could not show meaningful relationships in the phylogeny of the genera, *Anoxybacillus*, due to the high level of sequence conservation and the short length examined (119 amino acids).

In this study, rep-PCR was also used for investigating the phylogenetic divergence within the thermophilic genus, *Anoxybacillus.* According to Rademaker and De Bruijn (1997), the banding patterns produced from the application of these methods contained an adequate number of bands, indicating that BOX-, ERIC-, and REP-PCR fingerprints are applicable for typing of the *Anoxybacillus* strains. Through comparison of the three methods, we found that the BOX-PCR method showed the highest genetic polymorphism, when compared with ERIC- and REP- fingerprints; BOX-PCR profiles were more distinct for the different strains, including a higher number of bands. A similar finding was documented by Adiguzel *et al*. (2009), who determined that the BOX-PCR method generated more informative results than REP- and ERIC-PCR for the studied *Geobacillus* and *Bacillus* strains.

rep-PCR profiles showed that 14 type strains produced different banding patterns. Furthermore, in rep-PCR, the strains within the same species showed the same profile as their type strains. Thus, it has been proved that rep-PCR fingerprinting is a reproducible tool for the genotypic differentiation of *Anoxybacillus* species. Some other studies reported similar results, indicating that rep-PCR genomic fingerprint techniques are highly sensitive in the discrimination of mesophilic and thermophilic species, such as for the genera *Bacillus* and *Geobacillus* (Mora *et al*., 1998; Guillaume-Gentil *et al*., 2002; Meintanis *et al*., 2008; Adiguzel *et al*., 2009). Their studies indicated that

rep-PCR is an effective method for differentiating *Bacillus* and *Geobacillus* strains.

This is the first report on rep-PCR and *rpoB* gene application as a phylogenetic analysis tool in *Anoxybacillus.* The results of this study show that the *rpoB* sequence of the genus *Anoxybacillus* is highly conserved within the species, and has diverged sufficiently between species to enable the identification of *Anoxybacillus* species by sequence comparisons of *rpoB*. Thus, the *rpoB* and rep-PCR analyses are rapid and reliable methods for the molecular typing of *Anoxybacillus* species.

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