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Identification of *Geobacillus stearothermophilus* by restriction digestion with *Alu*I of the amplified 16S rDNA

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We present an easy, fast and accurate method for the identification of *Geobacillus stearothermophilus* – the type species of the genus *Geobacillus*. We used 16S rRNA gene restriction analysis with *Alu*I and demonstrated a discriminate restriction profile of this species. The presence of a fragment 162 bp in size and the absence of the 76 bp and 86 bp fragments was identified to be characterictic of the species *G. stearothermophilus*. For the further validation of the applicability of restriction analysis for the species identification, *G. stearothermophilus* DSM 13240 as well as environmental thermophilic endospore-forming isolates 3, 9, 17, 30, 31, 32A, 35C and 36A were examined. Restriction profiles of all the environmental isolates were identical to that of *G. stearothermophilus* DSM 22^T. The restriction pattern of DSM 13240 differed from these profiles, suggesting that this strain does not belong to the species *G. stearothermophilus*. The potential of 16S rRNA gene restriction analysis using *Alu*I for identification of the other species of geobacilli is limited.

Key words: *Geobacillus*, thermophilic bacteria, taxonomy, identification, 16S rDNA, restriction analysis

INTRODUCTION

Thermophilic endospore-forming bacteria attract much interest over the past decades. These bacteria are associated with heat-treated foods and, being not pathogenic, can cause food spoilage by producing acids and thermostable enzymes. On the other hand, thermophilic endospore-formers constitute an excellent resource of biotechnologically important products. Therefore, the development of classification and identification schemes of these important bacteria is a relevant field of modern taxonomy.

The genus *Geobacillus* represents aerobic or facultatively anaerobic, neutrophilic, obligately thermophilic, endospore-forming bacteria [1]. Today, there are 17 validly described species in the genus *Geobacillus*. Identification of these species by traditional biochemical techniques is imprecise and time-consuming. The significance of application of rDNA-based fingerprinting methods (amplified ribosomal DNA restriction analysis (ARDRA), ribosomal intergenic spacer analysis (RISA)) in this systematic group has been reported [2–6]. ARDRA proved to be a valuable technique for a rapid grouping of geobacilli. Restriction endonucleases *Alu*I, *Cfo*I *Hae*III, *Hinf*I, *Mse*I, *Rsa*I, *Taq*I were used for the genotyping of geobacilli [2–4, 6– 8].

In this study, we present an easy, fast and accurate method for the identification of *Geobacillus stearothermophilus* – the type species of the genus *Geobacillus*. We used 16S rDNA restriction analysis with *Alu*I and demonstrated the discriminate restriction profile of this species. This technique was validated with both the type strain and the thermophilic aerobic endospore-forming environmental isolates.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. Bacterial strains used in this work are listed in Table. The cultures were cultivated and maintained on nutrient agar. The bacterial genomic DNA was extracted from a fresh cell culture (after cultivation on nutrient agar for 14 h at 60 °C) using the Genomic DNA Purification Kit (Fermentas) according to the manufacturer's instructions.

Amplification of 16S rDNA. Universal bacterial primers 27F (GAG AGT TTG ATC CTG GCT CAG) and 1495R (CTA CGG CTA CCT TGT TAC GA) were used for amplification of 16S rDNA [9]. 16S rDNA was amplified in 50 µl of reaction mixture containing PCR buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 µM each primer, 1.25 U recombinant *Taq*DNA Polymerase and 10 ng of bacterial genomic DNA. The reaction mixture was supplemented with 10% (v/v) DMSO. Amplification was conducted under the following conditions: initial denaturation at 95 °C for 2 min followed by 29 cycles each consisting of 95 °C for 1 min, 50 °C for 2 min and 72 °C for 3 min with a final extension step at 72 °C for 7 min in an Eppendorf thermal cycler. Products of amplification were analysed by electrophoresis through 1% agarose gel. 16S rDNA PCR experiments were repeated three times using different DNA extractions for amplification.

Amplified 16S rDNA restriction analysis (ARDRA). Products from PCR primed by 27F/1495R were analysed by single enzyme digestion according to endonuclease manufacturer's instructions with *Alu*I (Fermentas). The restriction profile was analysed by electrophoresis through 5% polyacrylamide gel. The restriction

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Table. **List of *Geobacillus* strains used in this study**

Species	Strains	Source	16S rDNA sequences
G. caldoxylosilyticus	DSM 12041^T	Dr. D. Mora	AF067651
G. debilis	DSM 16016^T	DSMZ	AJ564616
G. gargensis	DSM 15378^T	DSMZ	AY193888
G. jurassicus	DSM 15726^T	DSMZ	AY312404
G. kaustophilus	DSM 7263^T	DSMZ	X60618
G. lituanicus	DSM 15325^T	VU DPPM	AY044055
G. pallidus	DSM 3670^T	DSMZ	Z26930
G. stearothermophilus	DSM 22^T	DSMZ	AB271757
	DSM 13240	DSMZ	
	3, 9, 17, 30, 31, 32A, 35C, 36A	VU DPPM	
G. subterraneus	DSM 13552^T	DSMZ	AF276306
G. tepidamans	DSM 16325^T	DSMZ	AY563003
G. thermocatenulatus	DSM 730^T	DSMZ	Z26926
G. thermodenitrificans	DSM 465^T	Dr. D. Mora	Z26928
G. thermoglucosidasius	DSM 2542^T	DSMZ	AB021197
G. thermoleovorans	DSM 5366^T	DSMZ	Z26923
G. toebii	DSM 14590^T	DSMZ	AF326278
G. uzenensis	DSM 13551^T	DSMZ	AF276306
G. vulcani	DSM 13174^T	DSMZ	AJ293805

DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; VU DPPM – Department of Plant Physiology and Microbiology, Vilnius University, Vilnius, Lithuania.

analysis was repeated three times using different DNA extractions for amplification and different amplification products for the restriction analysis. Results of these completely independent experiments were identical.

Restriction analysis *in silico*. The 16S rDNA sequences of the test strains were aligned using the MEGA 3.1 program [10]. The putative restriction maps were drawn and the exact sizes of the restriction fragments were determined using the SEQBUILDER component of LASERGENE 6 (DNASTAR).

RESULTS AND DISCUSSION

According to the literature data, restriction endonuclease *Alu*I is the most frequently used enzyme for ARDRA in the genus *Geobacillus* [2, 3, 6–8, 11, 12], notwithstanding some reports [6, 11, 12] that *Hae*III and *Cfo*I are the most useful restrictases for species and strain discrimination in the genus *Geobacillus* – these enzymes produced the highest number of differentiating bands. Our previous work [8] suggested that ARDRA with *Alu*I could be used for *Geobacillus lituanicus* identification in association with other restriction enzymes. In the current study, we tested the possibility to use ARDRA with *Alu*I for the identification of the other species of the genus *Geobacillus* and particularly for the identification of *G. stearothermophilus,* the type species of this genus.

In order to reveal the recognition sites of *Alu*I, 16S rDNA sequences of the type strains of all validly described species of the genus *Geobacillus* were aligned and putative restriction maps were constructed (data not shown). 5–8 *Alu*I restriction sites were identified in the sequences examined. Three restriction sites were common for all the sequences of our dataset (data not shown). Two restriction sites, common to the majority of the sequences, were not found in those of *G. stearothermophilus* (Fig. 1a) and *Geobacillus pallidus* (Fig. 1d). The other two sites were not found in four (Fig. 1b) and three (Fig. 1e) sequences.

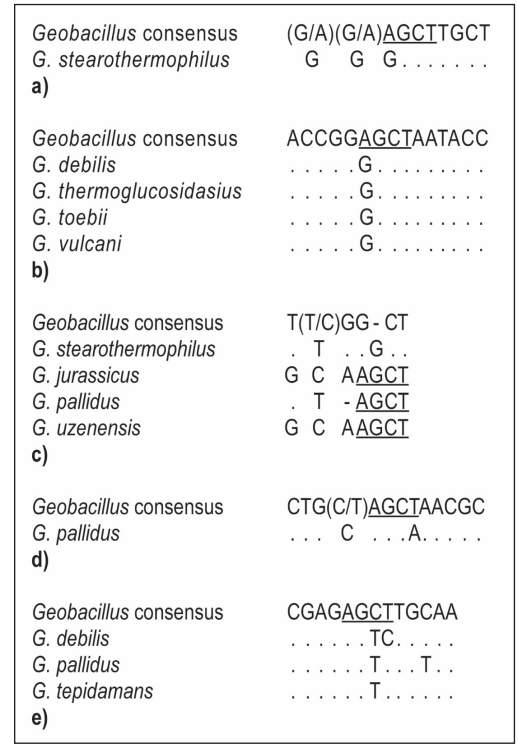

Fig. 1. Differentiation of *Alu*I restriction sites in 16S rRNA genes of geobacilli. The specific recognition sequence of *Alu*I is underlined. Dots indicate nucleotides identical to those of *Geobacillus* consensus sequence. Sequence gaps for the alignment are shown by hyphens. *G – Geobacillus*

The sequences of three species possessed an additional restriction site that was not found in those of the other species (Fig. 1c). On the basis of these results, it was hypothesized that *Alu*I restriction analysis could be useful for identification of some species of the genus *Geobacillus* (*G. stearothermophilus*, *G. debilis*, *G. jurassicus*, *G. pallidus*, *G. tepidamans*, *G. thermoglucosidasius*, *G. toebii*, *G. uzenensis*, *G. vulcani*) without using another restriction endonucleases.

It is known that rRNA genes are often organised as part of a multigene family, with the copy number ranging from 1 to 15 [13]. Sequence heterogeneity exists among multiple 16S rRNA genes encoded on a single genome [14]. Consequently, the putative restriction patterns of these distinct genes can differ. Therefore, it is recommended to compare the putative digestion profiles with those obtained experimentally [3]. We used genomic DNA for the amplification of 16S rDNA as it is believed that 16S rRNA genes of all the ribosomal operons can be amplified in such an experiment. We could not obtain 16S rDNA PCR product only for *G. debilis* DSM 16016^T because of the discrepancies in the binding region of the reverse primer 1495R (data not shown). All the other amplifications were successful. 16S rDNA PCR products were subjected to restriction analysis with *Alu*I. Seven different electrophoretic restriction profiles were obtained.

Gel-electrophoretic profiles of *Geobacillus caldoxylosilyticus* DSM 12041^T (Fig. 2, lane 2), *Geobacillus gargensis* DSM 15378^T (Fig. 2, lane 3), *Geobacillus kaustophilus* DSM 7263^T (Fig. 2, lane 4), *G. lituanicus* DSM 15325^T (Fig. 2, lane 5), *Geobacillus thermocatenulatus* DSM 730^T (Fig. 2, lane 6), *Geobacillus thermoleovorans* DSM 5366^T (Fig. 2, lane 8) and *G. vulcani* DSM 13174^T (Fig 2, lane 9) were identical irrespective of the lack of one *Alu*I recognition sequence in the 16S rRNA gene of the latter species (Fig. 1b). Consequently, all these species could not be identified on the basis of *Alu*I restriction analysis alone. Similarly, *Alu*I restriction profiles of *Geobacillus subterraneus* DSM 13552^T (Fig. 2, lane 10) and *G. uzenensis* DSM 13551^T (Fig. 2, lane 11) were identical, although the 16S rRNA gene sequence of the type strain of *G. uzenensis* showed an additional restriction site (Fig. 1c).

Results of *Alu*I restriction analysis were identical for *G. toebii* DSM 14590^T (Fig. 2, lane 14) and *G. thermoglucosidasius* DSM 2542^T (Fig. 2, lane 13). According to the putative restriction pattern of 16S rRNA genes of these species (Fig. 1b), they lacked one restriction site and this resulted in the absence of the fragment in the size of 86 bp (Fig. 2).

Only four species (*G. tepidamans*, *G. pallidus*, *G. jurassicus* and *G. stearothermophilus*) showed unique restriction profiles. It should be noted that *Alu*I restriction patterns of *G. tepidamans* DSM 16325^T (Fig. 3, lane 10) and *G. pallidus* DSM 3670^T (Fig. 3, lane 11) differed markedly from the patterns of the other species of the genus *Geobacillus*. We included the latter two species into our experiments as they were validly described as species of the genus *Geobacillus*. But these two species were predicted by us to belong to other genera, not *Geobacillus*, based on 16S rDNA phylogenetic analysis and genus-specific GEOBAC-PCR [15]. Hence, ARDRA with *Alu*I again indirectly proved our assumptions and demonstrated the distinctions of restriction profiles of these species.

The restriction pattern of *G. jurassicus* DSM 15726^T (Fig. 2, lane 12) differed from those of the other geobacilli in the presence of fragments 55 and 33 bp in size. These two fragments originated from the additional *Alu*I restriction site (Fig. 1c). Of course, the usefulness of ARDRA with *Alu*I should be tested in future with a larger number of *G. jurassicus* strains.

G. stearothermophilus DSM 22^T showed a unique *Alu*I restriction pattern – it was also predicted by the putative restriction profile (Fig. 2, lane 1). The presence of the fragment 162 bp in size and the absence of the fragments 76 bp and 86 bp in size were identified of be characterictic to the species *G. stearothermophilus*. For the further validation of the applicability of restriction analysis for species identification, strain DSM 13240 as well as the environmental thermophilic endospore-forming

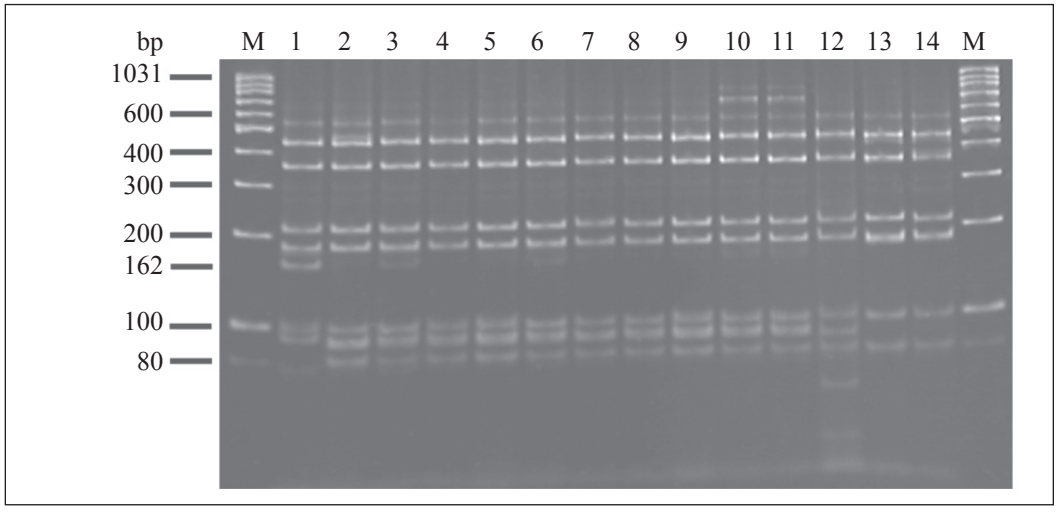

Fig. 2. ARDRA (*Alu*I) gel-electrophoretic profiles. For ARDRA, 16S rDNA was amplified in PCR primed by oligonucleotide pair 27F/1495R. *G – Geobacillus*. Lanes: 1 – *G. stearothermophilus* DSM 22^T, 2 – *G. caldoxylosilyticus* DSM 12041^T, 3 – *G. gargensis* DSM 15378^T, 4 – *G. kaustophilus* DSM 7263^T, 5 – *G. lituanicus* DSM 15325^T, 6 – *G. thermocatenulatus* DSM 730^T, 7 – *G. thermodenitrificans* DSM 465^T, 8 – *G. thermoleovorans* DSM 5366^T, 9 – *G. vulcani* DSM 13174^T, 10 – *G. subterraneus* DSM 13552^T, 11 – *G. uzenensis* DSM 13551^T, 12 – *G. jurassicus* DSM 15726^T, 13 – *G. thermoglucosidasius* DSM 2542^T, 14 – *G. toebii* DSM 14590^T, lane M, GeneRuler™ 100 bp DNA Ladder (Fermentas)

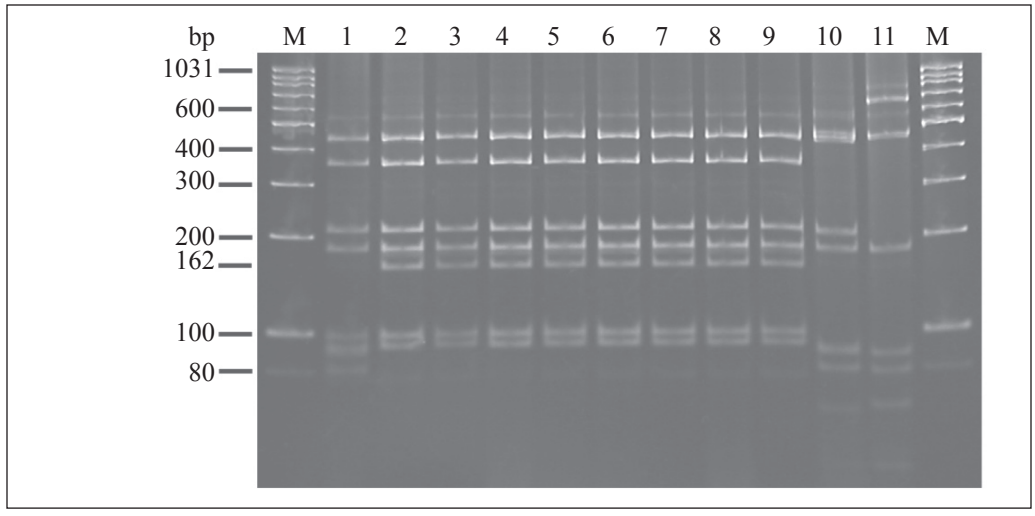

Fig. 3. ARDRA (*Alu*I) gel-electrophoretic profiles. For ARDRA, 16S rDNA was amplified in PCR primed by oligonucleotide pair 27F/1495R. *G – Geobacillus*. Lanes: 1 – *G. stearothermophilus* DSM 13240, 2 – *G. stearothermophilus* 3, 3 – *G. stearothermophilus* 9, 4 – *G. stearothermophilus* 17, 5 – *G. stearothermophilus* 30,; 6 – *G. stearothermophilus* 31, 7 – *G. stearothermophilus* 32A, 8 – *G. stearothermophilus* 35C, 9 – *G. stearothermophilus* 36A, 10 – *G. tepidamans* DSM 16325^T, 11 – *G. pallidus* DSM 3670^T, lane M, GeneRuler^{TM} 100 bp DNA Ladder (Fermentas)

isolates 3, 9, 17, 30, 31, 32A, 35C and 36A were examined. These environmental isolates were earlier identified as *G. stearothermophilus* by DNA-DNA hybridization (unpublished results). Results of ARDRA matched perfectly those of the hybridization: restriction profiles of all the environmental isolates were identical with that of *G. stearothermophilus* DSM 22^T (Fig. 3, lanes 2–9).

The restriction pattern of *G. stearothermophilus* DSM 13240 differed from the profiles of both the type strain of the species and the environmental isolates (Fig. 3, lane 1). It matched the profile of *G. caldoxylosilyticus* DSM 12041^T, *G. gargensis* DSM 15378^T, *G. kaustophilus* DSM 7263^T, *G. lituanicus* DSM 15325^T, *G. thermocatenulatus* DSM 730^T, *G. thermodenitrificans* DSM 465^T, *G. thermoleovorans* DSM 5366^T and *G. vulcani* DSM 13174^T. Strain DSM 13240 was predicted by us not to belong to the species *G. stearothermophilus* based on 16S rDNA phylogenetic analysis and genus-specific GEOBAC-PCR [15]. Hence, the results of ARDRA with *Alu*I were in concordance with results of the phylogenetic analysis as well as genus-specific GEOBAC-PCR. It should be noted here that the genome of this strain is under sequencing as the genome of the species *G. stearothermophilus*. The exact taxonomic position of this strain must be determined as postgenomic studies could lead to misinterpretations in future.

In conclusion, ARDRA using *Alu*I was shown to be a valuable, easy and accurate technique for the identification of *G. stearothermophilus*, the type species of the genus *Geobacillus*. It was validated with both the type strain and the thermophilic aerobic endospore-forming environmental isolates. The presence of the fragment 162 bp in size and the absence of the fragments 76 bp and 86 bp in size were identified to be characterictic of the species *G. stearothermophilus*. Of course, this species identification scheme should be tested in future with a larger number of strains in order to prove the reliability of proposed method of identification. The potential of ARDRA using *Alu*I in the identification of the other species of geobacilli is limited.

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GEOBACILLUS STEAROTHERMOPHILUS IDENTIFIKAVIMAS NAUDOJANT PAGAUSINTOS 16S rDNR RESTRIKCIJĄ *Alu*I RESTRIKTAZE

S a n t r a u k a

Šiame straipsnyje aprašome mūsų sukurtą nesudėtingą, greitą ir tikslų metodą *Geobacillus stearothermophilus*, tipinei *Geobacillus* genties rūšiai, identifikuoti. Šios rūšies 16S rDNR seką sukarpius *Alu*I restriktaze buvo gautas unikalus restrikcijos profilis. Palyginus jį su kitų rūšių 16S rDNR sekų restrikcijos rezultatais nustatyta, kad *G. stearothermophilus* profilis turėjo 162 bp dydžio fragmentą, kurio nebuvo kitų rūšių profiliuose, tačiau jame nesimatė tiems profiliams būdingų 76 bp ir 86 bp dydžio fragmentų. Siekiant įsitikinti, ar šis unikalus profilis būdingas ir kitiems *G. stearothermophilus* kamienams, buvo atlikta iš aplinkos išskirtų termofilinių endosporas formuojančių kamienų 3, 9, 17, 30, 31, 32A, 35C ir 36A bei *G. stearothermophilus* laboratorinio kamieno DSM 13240 16S rRNR geno restrikcija *Alu*I restriktaze. Visų iš aplinkos išskirtų kamienų restrikcijos profiliai sutapo su tipinio kamieno *G. stearothermophilus* DSM 22^T restrikcijos profiliu, o DSM 13240 kamieno profilis skyrėsi. Remdamiesi tyrimo rezultatais darome išvadą, kad DSM 13240 kamienas nepriklauso *G. stearothermophilus* rūšiai. Taip pat nustatėme, kad galimybė identifikuoti kitas *Geobacillus* genties rūšis naudojant 16S rRNR geno restrikcinę analizę *Alu*I restriktaze yra labai ribota.