Identification of the genus Geobacillus using genus-specific primers, based on the 16S–23S rRNA gene internal transcribed spacer

Nomeda Kuisiene, Juozas Raugalas, Milda Stuknyte & Donaldas Chitavichius

Department of Plant Physiology and Microbiology, Vilnius University, Vilnius, Lithuania

Correspondence: Nomeda Kuisiene, Department of Plant Physiology and Microbiology, Vilnius University, Chiurlionio 21/27, Vilnius LT-03101, Lithuania. Tel.: +370 5 2398205; fax: +370 5 2398204; e-mail: nomeda.kuisiene@gf.vu.lt

Received 18 June 2007; accepted 12 September 2007. First published online 26 October 2007.

DOI:10.1111/j.1574-6968.2007.00954.x

Editor: Clive Edwards

Keywords
Geobacillus; genus-specific primer; 16S–23S rRNA gene internal transcribed spacer; GEOBAC.

Abstract
The aim of this study was to develop an easy and accurate technique for the identification of the genus Geobacillus. For this purpose, Geobacillus genus-specific primers GEOBAC (GEOBAC-F and GEOBAC-R) based on the 16S–23S rRNA gene internal transcribed spacer (ITS) region sequences have been designed. In total, 52 sequences from three species of the genus Geobacillus (Geobacillus stearothermophilus, Geobacillus kaustophilus and Geobacillus lituanicus) were examined for the design of these primers. Analysis of the sequences revealed three highly conservative regions common to these species: 5’ and 3’ end regions of 16S–23S rRNA gene ITSs and box A. Some sequences possessed two additional conservative regions – genes of tRNAIle and tRNAAla. These particular sequences were chosen for the construction of the primers. The designed primers targeted the gene of tRNAIle and 3’ end region of ITSs. This technique was validated with both the reference strains of the genus Geobacillus and the thermophilic aerobic endospore-forming environmental isolates. Different Geobacillus species could be grouped according to the number and size of GEOBAC-PCR products and identified on the basis of the AluI and TaqI restriction analysis of these products.

Introduction
Taxonomy – and especially identification – of thermophilic endospore-forming bacteria have generated considerable interest over the past decades. These bacteria are associated with heat-treated foods, and, although they are not pathogenic, they can cause food spoilage by producing acids and thermostable enzymes (Mora et al., 1998). On the other hand, thermophilic endospore-formers constitute an excellent resource of biotechnologically important products: amylases (Mamo et al., 1999), proteases (Hawumba et al., 2002), pullulanases (Ben Messaoud et al., 2002), gellan lyases (Derekova et al., 2006), lipases (Soliman et al., 2007), carboxylesterases (Liu et al., 2007), L-arabinose isomerases (Rhimi et al., 2007), etc.

The genus Geobacillus represents aerobic or facultatively anaerobic, neutrophilic, obligately thermophilic, endospore-forming bacteria (Nazina et al., 2001). Identification of these species by traditional biochemical techniques is imprecise and time consuming. A few DNA-based techniques were suggested for rapid, easy and reliable genus and species identification of geobacilli. The potential application of recN sequence similarity for the identification of species within the genus Geobacillus was evaluated recently (Zeigler, 2005). Geobacillus genus-specific primer LEVO, based on the 16S rRNA gene sequences, was designed by Flint et al. (2001). Nevertheless, separate species could not be differentiated with this primer. Restriction analysis of 16S rRNA gene proved to be a valuable technique for rapidly grouping of geobacilli (Blanc et al., 1997; Mora et al., 1998; Caccamo et al., 2001; Kuisiene et al., 2002; Rahman et al., 2004). However, the use of this technique in the identification of the species is limited. Furthermore, it cannot be used for the genus identification.

16S–23S rRNA gene internal transcribed spacer (ITS) separates 16S and 23S rRNA genes and may contain tRNA genes. The sequence of ITS exhibits greater variations than that of the 16S rRNA structural gene. This variation can occur between species in both the length and the sequence of this region. Hence, ITS sequences are more useful for the genus- and species-specific primer design than 16S rRNA gene (Rachman et al., 2003; Kwon et al., 2004). The aim of the present study was to develop an easy and accurate technique for the identification of the genus Geobacillus.
For this purpose, genus-specific primers GEOBAC based on the ITS sequences have been designed. This technique was validated with both the reference strains and the thermophilic aerobic endospore-forming environmental isolates. The usefulness of the restriction analysis of the GEOBAC-PCR product for the species identification was also evaluated.

Materials and methods

Bacterial strains and DNA extraction

The bacterial strains used in this work are listed in Table 1. The cultures were cultivated and maintained on nutrient agar. The bacterial genomic DNA was extracted from 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.

Ribosomal intergenic spacer analysis (RISA)

RISA was performed with the primers S-D-Bact-1494-a-S-20 and L-D-Bact-0035-a-A-15 (Daffonchio et al., 1998) as described by Kuisiene et al. (2002). RISA profiles were analysed by electrophoresis through 1% agarose and 5% polyacrylamide gels. RISA experiments were repeated three times using different DNA extractions for amplification. The results of these completely independent experiments were identical.

Table 1. List of strains used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. caldoxylosilyticus</td>
<td>DSM 12041&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Dr. D. Mora</td>
</tr>
<tr>
<td>G. debilis</td>
<td>DSM 16016&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td>G. gargentis</td>
<td>DSM 15378&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td>G. jurassicus</td>
<td>DSM 15726&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td>G. kaustophilus</td>
<td>DSM 7263&lt;sup&gt;T&lt;/sup&gt;</td>
<td>HTA426</td>
</tr>
<tr>
<td>G. lituanicus</td>
<td>DSM 15325&lt;sup&gt;T&lt;/sup&gt;</td>
<td>VU DPPM</td>
</tr>
<tr>
<td>G. pallidus</td>
<td>DSM 3670&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td>G. stearotherophilus</td>
<td>DSM 22&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td></td>
<td>DSM 13240</td>
<td>DSMZ</td>
</tr>
<tr>
<td></td>
<td>3, 9, 17, 28, 30, 31, 32A, 36A</td>
<td>VU DPPM</td>
</tr>
<tr>
<td>G. subterraneus</td>
<td>DSM 13552&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td>G. tepidamans</td>
<td>DSM 16325&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td>G. thermocatenulatus</td>
<td>DSM 730&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td>G. thermodenitrificans</td>
<td>DSM 465&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Dr. D. Mora</td>
</tr>
<tr>
<td>G. thermoglucosidasi</td>
<td>DSM 2542&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td>G. thermoleovorans</td>
<td>DSM 5366&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td>G. toebii</td>
<td>DSM 14590&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td>G. uzenensis</td>
<td>DSM 13551&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
<tr>
<td>G. vulcani</td>
<td>DSM 13174&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSMZ</td>
</tr>
</tbody>
</table>

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

Construction of Geobacillus genus-specific primers based on the sequences of 16S–23S rRNA gene ITS

Strains Geobacillus stearothermophilus 3, G. stearothermophilus 28 and G. stearothermophilus 36A as well as Geobacillus lituanicus DSM 15325<sup>T</sup> were subjected to RISA analysis. Discrete fragments of RISA profile were extracted from agarose gel using the DNA Extraction Kit (Fermentas). The fragments were cloned and sequenced as described by Kuisiene et al. (2004). The sequences obtained were used for further analysis. Sequences of Geobacillus kaustophilus ATCC 8005<sup>T</sup> and G. stearothermophilus ATCC 12980<sup>T</sup> (AF478063 and AF478064, respectively), available in GenBank, were also used. ITS sequences were also extracted from the complete genome of G. kaustophilus HTA426 and from the contigs of the incomplete sequenced genome of G. stearothermophilus DSM 13240. As a consequence, the variability of ITS sequences between the different strains of the same species and between the different operons of the same strain could be estimated. ITS sequences of the other geobacilli could not be found in the public databases.

The sequences were edited and PCR primers were designed using the SEQUENCE and PRIMERSIM components of LASERGENE 6 (DNASTAR). Sequences were aligned and analysed using MEGA 3.1 program (Kumar et al., 2004). Identification of the genes of tRNA in these sequences was performed using the TRNASCAN-SE 1.21 program (Lowe & Eddy, 1997).

Amplification of ITS using Geobacillus genus-specific primers: GEOBAC-PCR

The designed primers GEOBAC-F and GEOBAC-R were used for the amplification of the ITS from the genomic DNA of all species of the genus Geobacillus and aerobic thermophilic environmental isolates. For amplification of ITS of Geobacillus toebii DSM 14590<sup>T</sup> and Geobacillus debilis DSM 16016<sup>T</sup>, the pair of primers GEOBAC-F and L-D-Bact-0035-a-A-15 (Daffonchio et al., 1998) was also used. The ITS region was amplified in 50 μL of reaction mixture containing PCR buffer with (NH₄)₂SO₄, 2 mM MgCl₂, 0.2 mM each dNTP, 0.25 μM each primer, 1.25 U recombinant TaqDNA Polymerase and 10 ng of bacterial genomic DNA. The reaction mixture was supplemented with 10% (v/v) dimethyl sulphoxide. 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, 60 °C (for GEOBAC) or 50 °C (for GEOBAC-F and L-D-Bact-0035-a-A-15) 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 5% polyacrylamide gel. GEOBAC-PCR experiments were repeated three times using different DNA extractions for amplification. The results of these completely independent experiments were identical.
Restriction analysis of GEOBAC-PCR products

Products of GEOBAC-PCR were analysed by single enzyme digestion according to the endonuclease manufacturer’s instructions with AluI, BsuRI, EcoRI, HaeII, PstI, PvuII and TaqI (Fermentas). The restriction profile was analysed by electrophoresis through 5% polyacrylamide gel. The restriction analysis was repeated three times using different DNA extractions for amplification and different amplification products for restriction analysis. The results of these completely independent experiments were identical.

Phylogenetic analysis of the 16S rRNA gene

The 16S rRNA gene sequences of the tested strains were aligned using the MEGA 3.1 program (Kumar et al., 2004). The size of the 16S rRNA gene used for alignment was 1361 nucleotides. A phylogenetic tree was constructed using the MEGA 3.1 program by the neighbour-joining method (Saitou & Nei, 1987). The pairwise-deletion option was used. Bootstrap analysis of the neighbour-joining data, using 1000 resamplings, was carried out in order to evaluate the validity and reliability of the tree topology. The tree was rooted using the X90640 sequence of Ureibacillus thermophilaericus DSM 10633T as an outgroup.

Results and discussion

Construction of Geobacillus genus-specific primers

In order to design Geobacillus genus-specific oligonucleotides, sequences, obtained after cloning and sequencing of ITS of Geobacillus stearothermophilus strains 3, 28, 36A and Geobacillus lituanicus DSM 15325T as well as the sequences available through the public databases, were analysed.

In total, 52 sequences from three species of the genus Geobacillus (Geobacillus stearothermophilus, Geobacillus kaustophilus and Geobacillus lituanicus) were used for the construction of GEOBAC primers (Table 2). Analysis of these sequences revealed 3 highly conservative regions common to these species: 5′ and 3′ end regions of ITS and box A (Fig. 1). Some sequences possessed two additional conservative regions – genes of tRNAIle and tRNAAla. These particular sequences were chosen for the construction of the primers. First, these sequences were longer than most of the sequences without the genes of tRNA. It is easier to analyse and, if needed, restriction analysis could be applied to larger PCR products.

Second, only one to two ITS sequences with the genes of tRNA for every strain examined were detected. Construction of primers based on the latter sequences allowed to reduce the number of the expected PCR products and minimize the probability of heteroduplex formation (Daffonchio et al., 2003).

The analysis showed that the best targets for the construction of the primers are the gene of tRNAIle and 3′ end region of ITS. Primers GEOBAC were designed. GEOBAC-F (TAA GCG TGA GGT CGG TGG TTC) targeted the gene of tRNAIle and 3′ end regions of ITS and box A (Fig. 1). Some

![Fig. 1. Schematic representation of conservative and variable regions of Geobacillus ITS. Boxes with diagonal lines represent variable regions of ITS; boxes with the lattice represent conservative regions; tRNA genes are shaded.](https://academic.oup.com/femsle/article-abstract/277/2/165/614476)
tRNA\(^{\text{Ile}}\), and GEOBAC-R (GGG CTC TCG GCT TCT TCC TT) targeted the 3′ end region of ITS.

**Validation of Geobacillus genus-specific GEOBAC primers**

To study whether the GEOBAC primers are appropriate for the identification of the genus Geobacillus, the type strains of all species of this genus were tested. Strains *G. kaustophilus* HTA426 (the genome of this strain has been sequenced by Takami et al., 2004) and *G. stearothermophilus* DSM 13240 (the genome of this strain is under sequencing in the University of Oklahoma) were also tested.

GEOBAC-PCR products were obtained for all the tested strains, except for *G. toebii* DSM 14590\(^T\), *G. debilis* DSM 16016\(^T\), *Geobacillus tepidamans* DSM 16325\(^T\) and *Geobacillus pallidus* DSM 3670\(^T\). The probable reasons for these unsuccessful amplifications are discussed below.

Three fragments were obtained for *Geobacillus thermoleovorans* DSM 5366\(^T\) (Fig. 2). Two different fragments were obtained for *Geobacillus vulcani* DSM 13174\(^T\) and *G. lituanicus* DSM 15325\(^T\). Different strains of *G. kaustophilus* (DSM 7263\(^T\) and HTA426) showed comparable GEOBAC-PCR electrophoretic profiles. The profiles of both strains consisted of two fragments, the smaller one being c. 380 bp in length. It is worth mentioning that the species *G. thermoleovorans*, *G. kaustophilus*, *G. lituanicus* and *G. vulcani* are very closely related and represent the phylogenetically recent species. This cluster of the species could be identified on the basis of the number of fragments in the GEOBAC-PCR.

The strains of other species demonstrated one distinct band on polyacrylamide gel (Fig. 2). Based on the fragment size, the latter strains could be subdivided into three clusters: (1) fragment size c. 400–420 bp in length (*Geobacillus thermocatenulatus* DSM 730\(^T\), *Geobacillus gargensis* DSM 15378\(^T\) and *G. stearothermophilus* DSM 13240); (2) fragment size c. 300–350 bp (*G. stearothermophilus* DSM 22\(^T\), *Geobacillus jurassicus* DSM 15726\(^T\), *Geobacillus subterraneus* DSM 13552\(^T\); lane 14, *Geobacillus thermodenitrificans* DSM 465\(^T\); lane 15, *Geobacillus caldoxylosilyticus* DSM 12041\(^T\) and 16, *Geobacillus thermoglucosidasius* DSM 2542\(^T\); lane M, GeneRuler\(^{TM}\) 100-bp DNA Ladder (Fermentas).

**Species identification in the genus Geobacillus based on restriction analysis of GEOBAC-PCR products**

The usefulness of the restriction analysis of the GEOBAC-PCR product for the species identification was analysed. The clusters of the species with a similar GEOBAC-PCR fragment size were chosen for this analysis. *Geobacillus jurassicus* DSM 15325\(^T\) was excluded from this experiment as this...
species could be easily identified based on the GEOBAC-PCR fragment size (c. 350 bp). *Geobacillus stearothermophilus* DSM 13240 was also excluded from this analysis based on the differences in the fragment size between this strain and the type strain of the species *G. stearothermophilus*. Aiming to verify their relatedness, the phylogenetic position of the strain DSM 13240 was evaluated (data are presented below).

Restriction analysis was performed with seven restriction enzymes: AluI, BsuRI, EcoRI, Hae II, PstI, PvuII and TaqI. The best resolution was achieved with AluI and TaqI (Fig. 3). *Geobacillus thermocatenulatus* DSM 730T and *G. gargensis* DSM 15378T were identical in AluI electrophoretic profiles but differed in TaqI patterns. *Geobacillus thermoglucosidasius* DSM 22T and *G. stearothermophilus* DSM 2542T could be easily identified based on both AluI and TaqI electrophoretic patterns. *Geobacillus subterraneus* DSM 13551T and *G. uzenensis* DSM 13551T could not be separated by whatever restriction enzyme was used. RISA was performed in order to investigate the genomic diversity of these two strains. The results of RISA are available online as supplementary Fig. S1. The identical RISA profiles of these two strains were surprising – this technique was reported to be strain- or species-specific (Flint et al., 2001; Rachman et al., 2003). According to the present results, two different species, belonging to the same genus, but remote phylogenetically, possessed identical RISA profiles. Therefore, the taxonomic positions of these two strains should be verified. The need for further analysis in order to confirm the taxonomic identity of the *G. uzenensis* strains was reported previously by Zeigler (2005).

For further validation of the applicability of the restriction analysis for the species identification, the thermophilic environmental isolates 9, 17, 30, 31 and 32A were also examined. GEOBAC-PCR product (c. 300 bp) was restricted using AluI and TaqI. Restriction profiles were identical for these five strains and matched perfectly the results for strain *G. stearothermophilus* DSM 22T (data not shown). Consequently, strains 9, 17, 30, 31 and 32A were identified as *G. stearothermophilus*. DNA–DNA hybridization experiments confirmed this conclusion.

In conclusion, the species exhibiting a single fragment size in GEOBAC-PCR (except for *G. subterraneus* and *G. uzenensis*) could be identified on the basis of restriction analysis and some of them (*G. jurassicus*) – on the basis of the fragment size. Of course, this species identification scheme should be tested in future with a larger number of strains, but the present results fairly suggest that GEOBAC-PCR products could be used for the species identification within the genus *Geobacillus*.

**Verification of the phylogenetic position of some strains of the genus *Geobacillus* by 16S rRNA gene analysis**

To ascertain the reasons for unsuccessful GEOBAC-PCR amplifications for some species of the genus *Geobacillus*, the 16S rRNA gene phylogenetic analysis was carried out. The taxonomic position of strain *G. stearothermophilus* DSM 13240 was also evaluated.

All thermophilic endospore-forming genera as well as the genus *Bacillus* were included in this analysis. The phylogenetic tree of the genus *Geobacillus* and the nearest neighbours is shown in Fig. 4. According to the analysis, *G. tepidamans* is related to the genus *Anoxybacillus* but not to *Geobacillus*. Close phylogenetic relationships between *G. tepidamans* and the genus *Anoxybacillus* have also been
shown by Poli et al. (2006). Geobacillus pallidus constituted a separate phylogenetic branch, not related to the genus Geobacillus. Hence, unsuccessful application of the GEOBAC primers to G. tepidamans DSM 16325 and G. pallidus DSM 3670 could be explained by the specificity of these primers to the target, i.e. ITS sequences of the genus Geobacillus.

GEOBAC-PCR products could not be obtained for G. debilis DSM 16016 and G. toebii DSM 14590 either. Amplification using the primers GEOBAC-F and L-D-Bact-0035-a-A-15 was successful for G. toebii DSM 14590, suggesting that some discrepancies in the region of binding of the GEOBAC-R primer are the reason for unsuccessful GEOBAC-PCR. In case of G. debilis DSM 16016, PCR product could not be obtained not only with the GEOBAC-F and L-D-Bact-0035-a-A-15 but also in the RISA experiment. It is supposed that some discrepancies with the GEOBAC primers as well as with the universal bacterial primers exist in the conservative regions of the genes of 16S and/or 23S rRNA gene and in the 3' end of ITS.

GEOBAC-PCR results for G. stearothermophilus DSM 13240 differed significantly from the results for the type strain of the species G. stearothermophilus. These results suggest that the strain does not belong to the species G. stearothermophilus. 16S rRNA gene phylogenetic analysis confirmed this assumption. The sequence of strain DSM 13240 was the most similar to those of G. kaustophilus HTA426 rrnA and rrnB, G. thermoleovorans DSM 5366 and G. vulcani DSM 13174 (99.3–99.6% sequence similarity). It should be noted 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 the postgenomic studies could lead to the misinterpretations in future.

In conclusion, GEOBAC primers, specific to the genus Geobacillus, were constructed based on ITS sequence. These primers could amplify the DNA from 13 Geobacillus species. Different species could be grouped according to the number and size of the PCR products and identified on the basis of the restriction analysis of these products. Application of GEOBAC primers also showed that the taxonomic position of some strains (G. stearothermophilus DSM 13240, G. subterraneus DSM 13552 and G. uzenensis DSM 13551) and species (G. tepidamans and G. pallidus) should be revisited.

Fig. 4. 16S rRNA gene phylogenetic tree of the genus Geobacillus and the nearest neighbours. The numbers at the nodes represent percent of bootstrap values obtained from 1000 samplings. Only the most significant values (higher than 70%) are presented. Ureibacillus thermosphae-ricus DSM 10633 was defined as an outgroup of the tree. Scale bar, 0.01 nucleotide substitution per site.
Acknowledgements

This work was supported by the grant of the Lithuanian State Science and Studies Foundation (project number T-053112). The authors are grateful to Dr Diego Mora (Milano University, Italy) and Dr Hideto Takami (Japan Agency of Marine-Earth Science and Technology, Japan) for Geobacillus strains.

Authors’ contribution

N.K. and J.R. contributed equally to this work.

References


---

**Supplementary Material**

The following supplementary material is available for this article:

**Fig. S1.** RISA profiles amplified in PCR primed by oligonucleotide pair S-D-Bact-1494-a-S-20/L-D-Bact-0035-a-A-15. Lane 1, *G. subterraneus* DSM 13552T; lane 2, *G. uzenensis* DSM 13551T; lane M, GeneRuler™ 100 bp DNA Ladder (Fermentas).

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2007.00954.x (This link will take you to the article abstract).

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.