Comparative sequence analysis of 16S-23S rRNA internal transcribed spacers of the genus Geobacillus

Nomeda Kuisienė*, Juozas Raugalas, Donaldas Čitavičius
Department of Plant Physiology and Microbiology, Vilnius University, M. K. Čiurlionio 21/27, Vilnius LT-03101, Lithuania

The aim of the present study was to analyse sequences of 16S-23S internal transcribed spacer regions of different Geobacillus species, identify structural elements of these regions and evaluate the possibility to use these structural elements in the taxonomy of the genus Geobacillus. GEOBAC-PCR was used in order to obtain 16S-23S rRNA internal transcribed spacer sequences. Certain structural elements were identified in these sequences: the genes of tRNA\textsubscript{Ile} and tRNA\textsubscript{Ala}, antitermination element boxA and variable regions of different length located between tDNA\textsubscript{Ala} and boxA (V1) as well as between boxA and the conservative 3’ end region of the spacer (V2). Sequence of the part of 3’ end region of 16S-23S internal transcribed spacers has been proved to be the genus signature region. A variable spacer between tRNA genes and region V1 as well as sequence of tRNA\textsubscript{Ala} gene carried signature information of species and species group.

Key words: Geobacillus, Geobacillus stearothermophilus, 16S-23S rRNA internal transcribed spacer, tRNA; GEOBAC

INTRODUCTION
Analysis of the PCR amplified 16S-23S rRNA internal transcribed spacer (ITS) was previously used for the genotyping of strains and identification of species within the genus Geobacillus [1–5]. Xu & Côté [6] carried out the phylogenetic analysis of this variable region of the two species – Geobacillus kaustophilus ATCC 8005\textsuperscript{T} and Geobacillus stearothermophilus ATCC 12980\textsuperscript{T}. The presence of the structural elements in these two sequences was not reported in the latter study.

It is generally accepted that most taxa of low G-C Gram-positive bacteria lack tRNA sequences in the ITS region, and, among those carrying the tRNA sequences, two tRNAs (tRNA\textsubscript{Ile} and tRNA\textsubscript{Ala}) are present in most species [7]. Two genes of tRNA (tDNA) were identified in some species of the endospore-forming bacterial genera: Bacillus, Brevibacillus, Tubribacillus, Sporolactobacillus [6–10]. It has been shown that ITS sequences containing tRNA genes are more informative for species discrimination than those without tDNA [8]. We have identified tRNA\textsubscript{Ile} and tRNA\textsubscript{Ala} genes in ITS of G. kaustophilus, G. stearothermophilus and Geobacillus lituanicus [11]. To our knowledge, tDNA has never been found in the ITS regions of other species of the genus Geobacillus. As far as we know, structural organization of ITS as well as interspecific sequence heterogeneity within the genus Geobacillus have never been analysed, either. The aim of the present study was to analyse sequences of ITS regions of different Geobacillus species, identify structural elements of the regions and evaluate the possibility to use these structural elements in the taxonomy of the genus Geobacillus.

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 fresh cell culture (after cultivation on nutrient agar for 14 hours at 60 °C) using the Genomic DNA Purification Kit (Fermentas) according to the manufacturer’s instructions.

Amplification of 16S-23S rRNA internal transcribed spacers
Geobacillus genus-specific primers GEOBAC were used for the amplification of ITS-containing genes of tRNA [11]. The target of GEOBAC-F is the fragment of tRNA\textsuperscript{Ile} gene, and that of GEOBAC-R – the conservative sequence in the 3’ end of the ITS. For the species from which the GEOBAC amplicons could not be obtained, the pair of primers GEOBAC-F and L-D-Bact-0035-a-A-15 [12] was used. The ITS region was amplified in 50 µl of a reaction mixture containing PCR buffer with (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 2 mM MgCl\textsubscript{2}, 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) of DMSO. Amplification was conducted in the following conditions: initial denaturation at 95 °C for 2 min, followed...
<table>
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<sup>a</sup> ITS sequences of these strains were obtained from the public databases.

<sup>b</sup> Sequences from Kuisiene et al. [11].

<sup>c</sup> Numbers of contigs are listed according to the data of 22-05-2007 available at http://www.genome.ou.edu/blast/bstearo_blastall.html

DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; VU DPPM – Department of Plant Physiology and Microbiology, Vilnius University, Vilnius, Lithuania; G – Geobacillus; B – Bacillus.
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 1% agarose gel.

Cloning and sequencing of ITS amplicons

GEOBAC-PCR amplicons obtained using genomic DNA of G. stearothermophilus DSM 7263\(^T\), Geobacillus thermocatenulatus DSM 5366\(^T\), Geobacillus vulcani DSM 13174\(^T\) and G. lituaniicus DSM 15325\(^T\) were extracted from agarose gel using the DNA Extraction Kit (Fermentas). The fragments were cloned and sequenced as described by Kuisiene et al. [13]. GEOBAC-PCR amplicons of Geobacillus thermocatenulatus DSM 730\(^T\), Geobacillus gargensis DSM 15378\(^T\), G. stearothermophilus DSM 22\(^T\), Geobacillus thermodenitrificans DSM 465\(^T\), Geobacillus subterraneus DSM 13552\(^T\), Geobacillus uzenensis DSM 13551\(^T\), Geobacillus caldoxylostylicus DSM 12041\(^T\), Geobacillus thermoglucosidasius DSM 2542\(^T\) and Geobacillus jurassicus DSM 15726\(^T\) were sequenced without cloning. PCR products obtained with the pair of primers GEOBAC-F and L-D-Bact-0035-a-A-15 using genomic DNA of Geobacillus toebii DSM 14590\(^T\), Geobacillus tepidamans DSM 16325\(^T\) and Geobacillus pallidus DSM 3670\(^T\) were also sequenced without cloning. The sequences obtained in the present study were deposited in the GenBank. Accession numbers of these sequences are listed in Table.

ITS sequences containing tRNA genes were also extracted from the whole genome sequences of G. stearothermophilus HTA426 and G. thermodenitrificans NG80-2 and from the contigs of the incomplete sequenced genome of G. stearothermophilus DSM 13240. Sequences of ITS containing tDNA of G. stearothermophilus strains 3, 28 and 36A as well as of other genera of Bacillaceae were also used for the analysis (Table).

The sequences were edited using the SEQBUILDER component of LASERGENE 6 (DNASTAR). The sequences were aligned and analysed using MEGA 3.1 program [14]. Identification of tRNA genes in these sequences was performed using the tRNAscan-SE 1.21 programme [15].

RESULTS AND DISCUSSION

Structural organization of ITS in the genus Geobacillus

It has been shown that ITS sequences containing tRNA genes are more useful for taxonomic purposes than those without tDNA [8]. Therefore, in our study, using GEOBAC primers we searched for such ITS – only sequences containing the gene of tRNA\(^{\text{Ala}}\) could be amplified in GEOBAC-PCR [11]. GEOBAC-PCR amplions were sequenced and applied for the sequence analysis.

Certain structural elements were identified in these sequences as well as in the sequences of G. kaustophilus HTA426, G. thermodenitrificans NG80-2 and G. stearothermophilus strains DSM 13240, 3, 28 and 36A. However, we could not identify some structural regions in the sequence of G. caldoxylostylicus DSM 12041\(^T\) because of the low quality of this sequence. The analysed sequences possessed two tRNA genes with a short spacer between them. The antitermination element (boxA) was also identified in all the examined sequences. Variable regions of different length were located between tDNA\(^{\text{Ala}}\) and boxA (V1) as well as between boxA and the conservative 3’ end region of ITS (V2). Hence, ITS containing tDNA were identical in terms of structural organization but differed in sequence that could be exploited for identification and grouping of taxa within the genus Geobacillus.

Geobacillus genus signature region of ITS containing tDNA

The part of 3’ end region of ITS was found to be conservative and was chosen for the design of the Geobacillus genus-specific primer GEOBAC-R [11]. Successful amplifications using this primer were achieved with all species of the genus Geobacillus except G. toebii DSM 14590\(^T\), Geobacillus debilis DSM 16016\(^T\), G. tepidamans DSM 16325\(^T\) and G. pallidus DSM 3670\(^T\). Amplification using GEOBAC-F and L-D-Bact-0035-a-A-15 resulted in PCR products for these species except G. debilis DSM 16016\(^T\). For G. toebii DSM 14590\(^T\) GEOBAC-R binding site had 3 mismatches with the sequence of the primer including 3’ terminal nucleotide, and this could be the reason for the unsuccessful application of GEOBAC-PCR to this species (Fig. 1). The sequences of G. thermodenitrificans NG80-2 showed two not terminal mismatches with the GEOBAC-R (G→A and one insertion). In contrast, sequences of the GEOBAC-R binding region of G. tepidamans DSM 16325\(^T\) and G. pallidus DSM 3670\(^T\) were considerably shorter than those of other geobacilli and markedly differed in sequence. Consequently, sequence analysis of the 3’ end region of ITS confirmed previous suggestions concerning an improper taxonomic position of both G. tepidamans DSM 16325\(^T\) and G. pallidus DSM 3670\(^T\) [11] and proved the GEOBAC-R binding region to be Geobacillus genus-specific.

Geobacillus species signature regions of ITS containing tDNA

1. tRNA\(^{\text{Ala}}\) genes in the sequences of ITS

The target of the primer GEOBAC-F was the tRNA\(^{\text{Ala}}\) gene. Successful application of this primer to all species of the genus Geobacillus except G. debilis DSM 16016\(^T\) demonstrated that
all species possess this gene in some of their ITS sequences. Sequencing of the GOBAC-PCR products confirmed this suggestion (Fig. 2). The gene of tRNA\textsubscript{Ala} was also identified in all the sequences examined. This gene was more variable than the tRNA\textsubscript{Ile} gene. The sequences of this gene of \textit{G. thermoleovorans} DSM 5366\textsuperscript{T}, \textit{G. kaustophilus} HTA426 \textsuperscript{rrn}B, \textit{G. stearothermophilus} 28 clone 05 and \textit{G. pallidus} DSM 3670\textsuperscript{T} differed from the other sequences in 1 (C\textsuperscript{→}T), 1 (G\textsuperscript{→}A), 1 (G\textsuperscript{→}A) and 2 (T\textsuperscript{→}G and A\textsuperscript{→}C) positions, respectively (Fig. 2). We could not identify 2 nucleotides in the sequences of \textit{G. caldoxylsilyticus} DSM 12041\textsuperscript{T} and \textit{G. gargensis} DSM 15378\textsuperscript{T}. It should be noted that according to the nucleotide present in position 38 (A or C) of the tRNA\textsubscript{Ala} gene, two distinct groups of the species could be identified. Only more phylogenetically recent species (\textit{G. kaustophilus}, \textit{G. thermoleovorans}, \textit{G. lituanicus}, \textit{G. vulcani}, \textit{G. thermocatenulatus} and \textit{G. gargensis}) as well as \textit{G. jurassicus} possessed A in this position.

In conclusion, all the analysed sequences contained a pair of tRNA\textsubscript{Ile} and tRNA\textsubscript{Ala} genes. Sequence analysis of the tRNA\textsubscript{Ala} gene could be useful for species grouping within the genus \textit{Geobacillus}.

2. Analysis of the variable spacer between the tRNA\textsubscript{Ile} and tRNA\textsubscript{Ala} genes

Spacers between the tRNA genes have been shown to be useful for the identification of grampositive bacteria species [8, 16]. Our results showed that some species of the genus \textit{Geobacillus} (\textit{G. jurassicus}, \textit{G. stearothermophilus}, \textit{G. thermodenitrificans}, \textit{G. thermoglucosidasius}, \textit{G. toebii}, \textit{G. pallidus}, \textit{G. tepidamans})
could be also identified on the basis of this region. These species differed in both the length and sequence of this spacer (Fig. 2). The most phylogenetically recent species (G. kaustophilus, G. thermoleovorans, G. lituanicus, G. vulcani, G. thermocatenulatus and G. gargensis) possessed the identical spacer TTGGAT. The other two species, G. subterraneus and G. uzenensis, had an identical spacer sequence, TCCTATATTGT. Consequently, the sequence of the spacer between tRNA genes could not be used for the identification of the species mentioned above, but it could be useful for the characterization of these two species groups. It should be noted that the strain G. stea rothermophilus DSM 13240 showed a spacer sequence different from the type strain G. stea rothermophilus DSM 22 and the other G. stea rothermophilus strains (Fig. 2). The sequence of DSM 13240 was identical with that of the G. kaustophilus – G. gargensis group. This strain was previously suggested not to belong to the species G. stea rothermophilus [11]. Analysis of the spacer between tRNA genes repeatedly confirmed this suggestion.

Our results have clearly showed that characteristics of the spacer between tRNA genes – both length and sequence – are useful for species and species group identification within the genus Geobacillus.

3. Variable region located between tRNA\^dt (V1)
In our study, the 5' end of V1 (8 bp in length) flanking the 3' end of tRNA\^dt gene was found useful for species and species group identification in the genus Geobacillus (Fig. 2). G. jurassicus, G. stea rothermophilus, G. thermodenitrificans, G. thermoglucosidasius, G. caldoxylosilyticus, G. toebii, G. tepidamans and G. pallidus could be identified on the basis of this short flanking fragment. In contrast, phylogenetically recent species (G. kaustophilus, G. thermoleovorans, G. lituanicus and G. vulcani) possessed an identical sequence, TATCAAGG, the only exception being G. lituanicus DSM 15325\(^T\) clone 36 (the flanking sequence TATCAAGT). Despite this difference, the consensus sequence of this group, TATCAAGG, differed from the other flanking sequences by at least two nucleotides and could be used as the characteristic of this species group. It should be mentioned that this short region of G. stea rothermophilus DSM 13240 was completely different from that of G. stea rothermophilus DSM 22 and was identical to the sequence of G. lituanicus DSM 15325\(^T\) clone 36. G. subterraneus and G. uzenensis as well as G. thermocatenulatus and G. gargensis could not be differentiated according to the analysis of this short variable region. But the latter four species could be identified as two groups of species because of the sequences different from those of the other Geobacillus species. The sequence of this short variable region could be useful for differentiating between G. thermocatenulatus – G. gargensis and G. kaustophilus – G. lituanicus – G. thermo leovorans – G. vulcani species groups – sequences of conservative regions as well as the spacers between the genes of tRNA were identical for these six species and could not differentiate them.

The remaining part of V1 was very variable both in length and sequence in G. kaustophilus – G. lituanicus – G. thermoleovorans – G. vulcani species group. Significant differences were detected not only between sequences of different strains of the same species, but even between different operons of G. lituanicus DSM 15325\(^T\) and G. kaustophilus HTA426 (data not shown). Consequently, although this part of V1 of different strains and different operons of G. stea rothermophilus and G. thermodenitrificans was conservative, we suppose that it should be used with caution for species identification and grouping.

ITS regions with no taxonomic significance
Three regions with no taxonomic significance were identified in Geobacillus ITS containing tDNA. Highly conservative antitermination elements, boxA sequences, were identified in all the examined sequences except G. caldoxylosilyticus DSM 12041\(^T\) because of the low quality of the latter sequence (Fig. 3). The boxA sequences of G. tepidamanas DSM 16325\(^T\) and G. pallidus DSM 3670\(^T\) differed from those of other Geobacillus species and were identical with the boxA sequences of the Bacillus cereus group, B. subtilis subsp. subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens and Lysinibacillus fusiformis. These two species of geobacilli have been recently supposed not to belong to the genus Geobacillus [11].

Fig. 3. Antitermination element boxA of the genus Geobacillus and other Bacillaceae genera. Antitermination element is boxed. Only sequences with tRNA genes were used for the alignment. Bacillus spp.: Bacillus subtilis subsp. subtilis, Bacillus cereus, Bacillus thuringiensis, Bacillus mycoides, Bacillus weihenstephanensis, Bacillus amyloliquefaciens, Bacillus licheniformis; Geobacillus spp.: G. gargensis, G. jurassicus, G. kaustophilus, G. lituanicus, G. stea rothermophilus, G. subterraneus, G. thermocatenulatus, G. thermodenitrificans, G. thermoglucosidasius, G. thermoleovorans, G. toebii, G. uzenensis, G. vulcani; Halobacillus spp.: Halobacillus litoralis, Halobacillus salinus, Halobacillus trueperii

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<td>ACTAGATAAC</td>
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<td>T . A . . . A . . . G A</td>
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Hence, according to our results, the sequence of the anti-
termination element was useful for revealing the incorrect
taxonomic position of both *G. tepidamanas* DSM 16325<sup>T</sup>
and *G. pallidus* DSM 3670<sup>T</sup>. On the other hand, the boxA
sequence of *Geobacillus* was identical with that of *Marinibacillus
marinus* (Fig. 3). So, this sequence could not be interpreted as the
*Geobacillus* genus signature region within the *Bacillaceae*
family.

The second region with no taxonomic significance was the
tRNA<sup>th</sup>. This gene is highly conservative among different
genera of endospore-formers (data not shown). The third
region was a variable fragment located between boxA and the
conservative 3′ end region of ITS (V2). This region was the most
variable part of ITS. Sequences of V2 varied in different strains
and different operons of *G. stearothermophilus*, *G. thermodenitrificans*,
*G. kaustophilus* and *G. lituanicus* (data not shown). Hence, these two regions could not be interpreted as the genus
or species signature regions.

In conclusion, tRNA<sup>th</sup>-tRNA<sup>al</sup> genes were identified in
ITS of all *Geobacillus* species except *G. debilis*. The sequence of
the part of 3′ end region of 16S-23S internal transcribed spacers
has been proven to be the genus signature region. Variable regions between tRNA genes and between tDNA<sup>th</sup> and boxA as
well as sequence of tDNA<sup>al</sup> carried the signature information of species and species group. Analysis of the sequences showed also that the taxonomic position of the strain *G. stearother-
ophilus* DSM 13240 and the species *G. tepidamanas* and *G. pal-
lidus* should be revised.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Lithuanian State
Science and Studies Foundation (project number T-05112). We
are thankful to Dr. Diego Mora (Milano University, Italy) for the
type strains of *G. thermodenitrificans* and *G. caldolyso-
lyticus*.

Received 28 September 2007
Accepted 3 February 2008

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