BIOLOGIJA. 2008. Vol. 54. Nr. 1. P. 1–6

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

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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 $tRNA^{Ile}$ and $tRNA^{Ala}$, antitermination element boxA and variable regions of different length located between $tDNA^{Ala}$ 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 $tRNA^{Ala}$ 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^T and *Geobacillus stearothermophilus* ATCC 12980^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 ($tRNA^{Ile}$ and $tRNA^{Ala}$) are present in most species [7]. Two genes of tRNA (tDNA) were identified in some species of the endospore-forming bacterial genera: *Bacillus, Brevibacillus, Tuberibacillus, 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 $tRNA^{Ile}$ and $tRNA^{Ala}$ 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*.

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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 $tRNA^{Ile}$ 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 $(NH_4)_2SO_4$, 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) of DMSO. Amplification was conducted in the following conditions: initial denaturation at 95 °C for 2 min, followed

Table. **List of strains and sequences used in this study**

Species	Strain	Source	Accession No. of 16S–23S rRNA ITS sequence
G. caldoxylosilyticus	DSM 12041[T]	Dr. D. Mora	EU157941
G. debilis	DSM 16016[T]	DSMZ	
G. gargensis	DSM 15378[T]	DSMZ	EU157942
G. jurassicus	DSM 15726[T]	DSMZ	EU157943
G. kaustophilus	DSM 7263[T]	DSMZ	EU157944, EU157945
	HTA426[a]		BA000043, rrnA ITS 11974–12605 BA000043, rrnB ITS 32344–33137
G. lituanicus	DSM 15325[T]	VU DPPM	EF645698[b], EF645700[b], EF645701[b], EU157946, EU157947
G. pallidus	DSM 3670[T]	DSMZ	EU157948
G. stearothermophilus	DSM 22[T]	DSMZ	EU157949
	DSM 13240[a, c]		Contig349, ITS 26519–27159
	3	VU DPPM	EF645707[b], EF645714[b]
	28	VU DPPM	EF645716[b], EF645724[b]
	36A	VU DPPM	EF645702[b], EF645704[b]
G. subterraneus	DSM 13552[T]	DSMZ	EU157950
G. tepidamans	DSM 16325[T]	DSMZ	EU157951
G. thermocatenulatus	DSM 730[T]	DSMZ	EU157952
G. thermodenitrificans	DSM 465[T]	Dr. D. Mora	EU157953
	NG80-2[a]		CP000557, ITS001 12169–12743 CP000557, ITS002 30880–31452
G. thermoglucosidasius	DSM 2542[T]	DSMZ	EU157954
G. thermoleovorans	DSM 5366[T]	DSMZ	EU157955
G. toebii	DSM 14590[T]	DSMZ	EU157956
G. uzenensis	DSM 13551[T]	DSMZ	EU157957
G. vulcani	DSM 13174[T]	DSMZ	EU157958
Virgibacillus marismortui	DSM 12325[Ta]		AB243786
Halobacillus salinus	JCM11546[Ta]		AB243776
Halobacillus litoralis	DSM 10405[Ta]		AB243772
Halobacillus trueperi	DSM 10404[Ta]		AB243769, AB243768
Lysinibacillus fusiformis	ATCC 7055[Ta]		AF478083
Lysinibacillus fusiformis	ATCC 7055[Ta]		AF478083
Marinibacillus marinus	ATCC 29841[Ta]		AF478075
B. cereus	DSM 31[Ta]		AJ420052
B. thuringiensis	DSM 2046[Ta]		AJ841875
B. mycoides	DSMZ 2048[Ta]		AJ420064
B. weihenstephanensis	WBC10204[Ta]		AJ420061
B. amyloliquefaciens	ATCC 23350[Ta]		AF478079
B. licheniformis	ATCC 14580[Ta]		AF478087
B. subtilis subsp. *subtilis*	168[a]		Z99104

[a] ITS sequences of these strains were obtained from the public databases.

[b] Sequences from Kuisiene et al. [11].

[c] 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. kaustophilus* DSM 7263^T, *Geobacillus thermoleovorans* DSM 5366^T, *Geobacillus vulcani* DSM 13174^T and *G. lituanicus* 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 caldoxylosilyticus* 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. kaustophilus* 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^{Ile} could be amplified in GEOBAC-PCR [11]. GEOBAC-PCR amplicons 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. caldoxylosilyticus* 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^{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^{Ala} genes in the sequences of ITS

The target of the primer GEOBAC-F was the tRNA^{Ile} gene. Successful application of this primer to all species of the genus *Geobacillus* except *G. debilis* DSM 16016T demonstrated that

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                                          1       10                  20       29
G. stearothermophilus 36A (EF645702)      CAAT|AAGGAAGAAGCCGAG -GCGC|TGTA
G. kaustophilus HTA426 (rrnA)             AGCA|...............-....|AA..
G. thermodenitrificans NG80-2 (ITS002)    ...C|..............A.C....|....
G. toebii                                 G.CA|T...............A...A|A.GC
G. pallidus                               ATG.|T.........A.----------|G..T
G. tepidamans                             ..TG|...-..-.....----------|.C.T
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Fig. 1. GEOBAC-R binding region in the 3' end of 16S-23S rRNA internal transcribed spacer. Dots indicate nucleotides identical to those of internal transcribed spacer of *Geobacillus stearothermophilus* 36A (acc. No. EF645702). Sequence gaps for the alignment are shown by hyphens. *G – Geobacillus*

all species possess this gene in some of their ITS sequences. Sequencing of the GEOBAC-PCR products confirmed this suggestion (Fig. 2). The gene of tRNA[Ala] was also identified in all the sequences examined. This gene was more variable than the tRNA[Ile] gene. The sequences of this gene of *G. thermoleovorans* DSM 5366[T], *G. kaustophilus* HTA426 rrnB, *G. stearothermophilus* 28 clone 05 and *G. pallidus* DSM 3670[T] differed from the other sequences in 1 (C→T), 1 (G→A), 1 (G→A) and 2 (T→G and A→C) positions, respectively (Fig. 2). We could not identify 2 nucleotides in the sequences of *G. caldoxylosilyticus* DSM 12041[T] and *G. gargensis* DSM 15378[T]. It should be noted that according to the nucleotide present in position 38 (A or C) of the tRNA[Ala] gene, two distinct groups of the species could be identified. Only more phylogenetically recent species (*G. kaustophilus, G. thermoeovorans, G. lituanicus, G. vulcani, G. thermocatenulatus* and *G. gargensis*) as well as *G. jurassicus* possessed A in this position. *G. stearothermophilus* DSM 13240 had A in this position, in contrast with the sequence of the type strain of *G. stearothermophilus* DSM 22[T] as well as other strains of *G. stearothermophilus* (C in this position) (Fig. 2).

In conclusion, all the analysed sequences contained a pair of tRNA[Ile] and tRNA[Ala] genes. Sequence analysis of the tRNA[Ala] gene could be useful for species grouping within the genus *Geobacillus*.

2. Analysis of the variable spacer between the tRNA[Ile] and tRNA[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 *Geobacillus* (*G. jurassicus, G. stearothermophilus*, *G. thermodenitrificans, G. thermoglucosidasius, G. toebii, G. pallidus, G. tepidamans*)

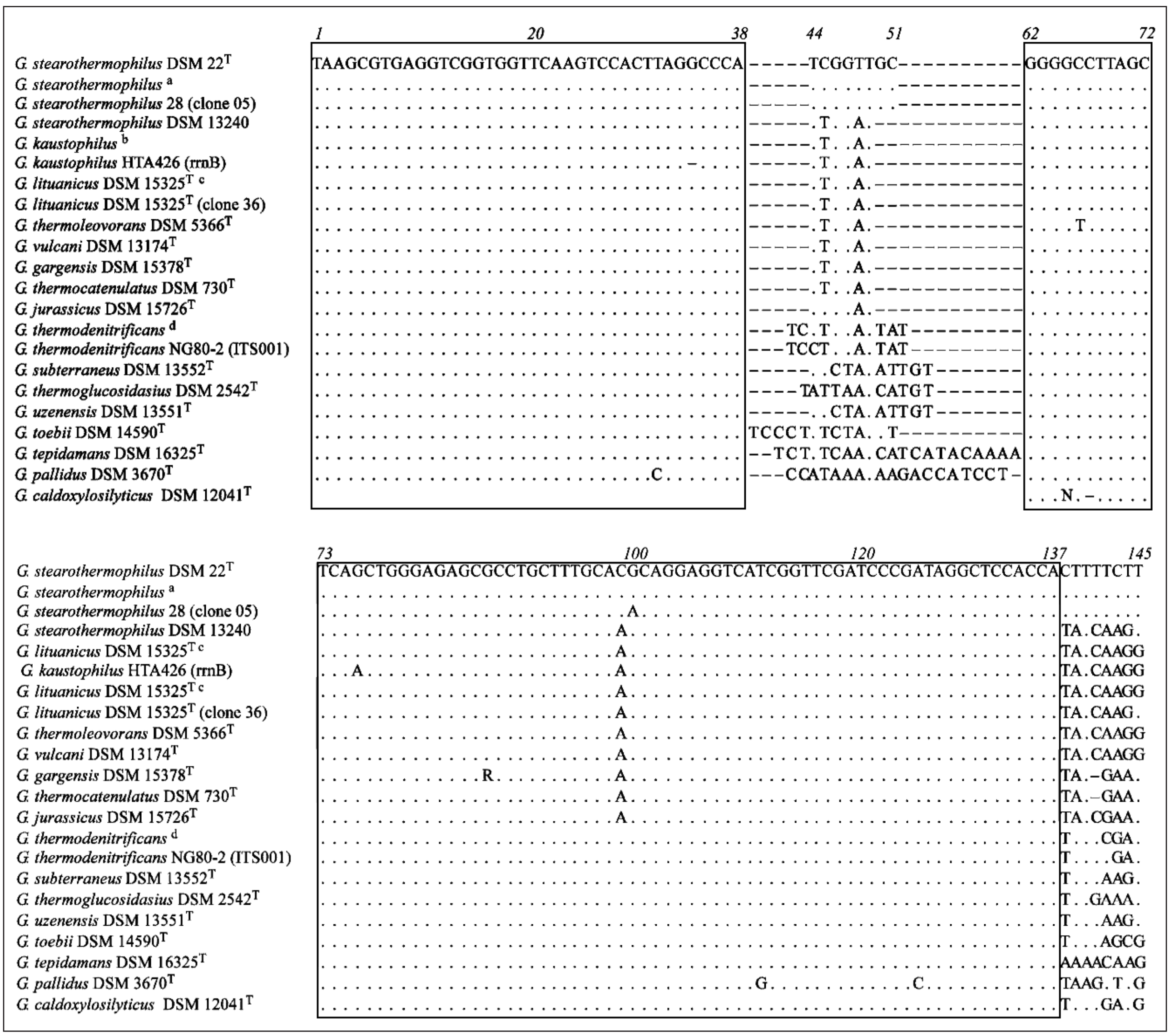

Fig. 2. Sequence alignment of tRNA genes (boxed), variable spacer between the genes and 5′ end of variable region V1 (8 bp in length) flanking the 3′ end of tRNA[Ala] gene. Dots indicate nucleotides identical to those of ITS of *Geobacillus stearothermophilus* DSM 22[T]. Sequence gaps for the alignment are shown by hyphens. *G. stearothermophilus*[a] – sequences EF645702, EF645704, EF645707, EF645714 and EF645724. *G. kaustophilus*[b] – sequences BA000043 (rrnA ITS 11974–12605), EU157944 and EU157945. *G. lituanicus* DSM 15325[Tc] – sequences EF645698, EF645701, EU157946 and EU157947. *G. thermodenitrificans*[d] – sequences CP000557 (ITS002 30880-31452) and EU157953

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. stearothermophilus* DSM 13240 showed a spacer sequence different from the type strain *G. stearothermophilus* DSM 22^T and the other *G. stearothermophilus* 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. stearothermophilus* [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^{Ala} gene and boxA (V1)

In our study, the 5' end of V1 (8 bp in length) flanking the 3' end of tRNA^{Ala} gene was found useful for species and species group identification in the genus *Geobacillus* (Fig. 2). *G. jurassicus, G. stearothermophilus, 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, TATCAAG(G/T), 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. stearothermophilus* DSM 13240 was completely different from that of *G. stearothermophilus* DSM 22^T 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. thermoleovorans – 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. stearothermophilus* 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].

	1	24
Geobacillus spp.	(C/G)G T T C C T T G A A A	A C T A G A T A A C C G
G. pallidus	T . . . . T	. . . G . . . . . . – .
G. tepidamans	T . . . . T	. . . . A .
Bacillus spp.	T . . . . T	. . . . A .
Lysinibacillus fusiformis	T . . . . T	. . . G . . . . . A A C
Marinibacillus marinus	C	. . . G . . . . . A G A
Halobacillus spp.	T . A A	. . . G . . . . . G (A/G)(A/G)
Virgibacillus marismortui	T . . A	. . . . A . . . . G A .

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. stearothermophilus, G. subterraneus, G. thermocatenulatus, G. thermodenitrificans, G. thermoglucosidasius, G. thermoleovorans, G. toebii, G. uzenensis, G. vulcani; Halobacillus* spp.: *Halobacillus litoralis, Halobacillus salinus, Halobacillus trueperi*

Hence, according to our results, the sequence of the antitermination element was useful for revealing the incorrect taxonomic position of both *G. tepidamanas* DSM 16325^{T} and *G. pallidus* DSM 3670^{T}. 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^{Ile} gene. 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^{Ile}-tRNA^{Ala} 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^{Ala} and boxA as well as sequence of tDNA^{Ala} carried the signature information of species and species group. Analysis of the sequences showed also that the taxonomic position of the strain *G. stearothermophilus* DSM 13240 and the species *G. tepidamans* and *G. pallidus* 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. caldoxylosilyticus*.

Received 28 September 2007
Accepted 3 February 2008

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PALYGINAMOJI TRANSKRIBUOJAMŲ SEKŲ, ESANČIŲ TARP *GEOBACILLUS* GENTIES BAKTERIJŲ 16S IR 23S rRNR GENŲ, ANALIZĖ

Santrauka

Buvo ištirtos transkribuojamos sekos (ITS), esančios tarp *Geobacillus* genties bakterijų 16S rRNR ir 23S rRNR genų, identifikuoti jų struktūriniai elementai ir įvertinta galimybė tuos struktūrinius elementus panaudoti *Geobacillus* genties taksonomijai. 16S–23S rRNR tarpgeninės srities sekos buvo gautos naudojant GEOBAC-PGR sistemą. Visose gautose sekose buvo nustatyti tokie struktūriniai elementai: tRNR^{Ile} ir tRNR^{Ala} genai, antiterminacijos elementas A bei dvi įvairaus ilgio variabilios sritys: tarp tRNR^{Ala} geno ir antiterminacijos elemento A sekų esanti variabilioji dalis V1 bei variabilioji dalis V2 tarp antiterminacijos elemento A ir ITS sekos pačiame 3' gale esančios konservatyvios dalies. Nustatyta, kad 3' gale esanti konservatyvioji sekos dalis yra svarbi nustatant bakterijų gentį. Rūšims ar rūšių grupėms identifikuoti svarbios yra variabilioji sekos dalis tarp abiejų tRNR genų, V1 dalis bei tRNR^{Ala} koduojanti seka.