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Geobacillus lituanicus sp. nov.

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Obligately thermophilic, aerobic, proteolytic, endospore-forming strain N-3^T was isolated from a high-temperature oilfield in Lithuania. 16S rRNA gene sequence analysis placed this strain in genetic group 5 of the endospore formers. *Geobacillus thermoleovorans* appeared to be the closest phylogenetic neighbour (99·4 % sequence similarity). The G+C content of strain N-3^T was 52·5 mol% and matched the range established for the genus *Geobacillus*. Studies of DNA–DNA relatedness and morphological and physiological analyses enabled strain N-3^T to be described as a member of the genus *Geobacillus*, but could not assign this strain to any other known species of this genus. Results of this polyphasic study allowed characterization of strain N-3^T as a novel species in the genus *Geobacillus* – *Geobacillus lituanicus* sp. nov. This species can be distinguished from *G. thermoleovorans* and *Geobacillus stearothermophilus* on the basis of 16S rRNA gene PCR-RFLP assays with the restriction endonucleases *Alu*I, *Hae*III and *Taq*I. The type strain of the novel species is N-3^T (=DSM 15325^T=VKM B-2294^T).

Over the last 10 years, much attention has been paid to the systematics of bacilli, particularly to the thermophilic bacilli of genetic group 5 (Ash *et al.*, 1991; Rainey *et al.*, 1994). Today, there are ten species with validly published names in the genus *Geobacillus* (Nazina *et al.*, 2001) embracing almost all of the 5th genetic group. Four of them have been described in the last 3 years: *Geobacillus subterraneus* (Nazina *et al.*, 2001), *Geobacillus uzenensis* (Nazina *et al.*, 2001), *Geobacillus caldoxylosilyticus* (Fortina *et al.*, 2001) and *Geobacillus toebii* (Sung *et al.*, 2002). It was shown that the novel species *Bacillus vulcani* also belongs to genetic group 5 (Caccamo *et al.*, 2000). The species name *Geobacillus thermodenitrificans* was validated recently (Manachini *et al.*, 2000; Nazina *et al.*, 2001). The names of the thermophilic species '*Bacillus caldotenax*', '*Bacillus caldovelox*' and '*Bacillus caldolyticus*' have not been validly published and are supposed to belong to the species *Geobacillus thermoleovorans* (Sunna *et al.*, 1997; Mora *et al.*, 1998). The latter species presumably embraces *Geobacillus thermocatenulatus* as well as *Geobacillus kaustophilus* (Sunna *et al.*, 1997).

All species of the genus *Geobacillus* are very closely related phylogenetically. Intragenic similarities for the 16S rRNA gene are more than 96·5 % (Nazina *et al.*, 2001). Quickly developing systematics for thermophilic endospore formers requires rigorous and informative methods for fast identification and grouping of strains. The significance of the application of rDNA-based fingerprinting methods (ARDRA and RSA) in this systematic group was reported previously (Blanc *et al.*, 1997; Mora *et al.*, 1998; Manachini *et al.*, 2000; Caccamo *et al.*, 2001; Fortina *et al.*, 2001). These methods are good alternatives to more laborious techniques, such as morphological and physiological analyses, currently used for screening and identification of strains.

Strain N-3^T was isolated from crude oil of the oilfield Girkaliai, which is located in Lithuania. The depth of sampling was 2000 m. The temperature of the oilfield was 60 °C and the pH was 6·5.

The isolation of thermophilic, aerobic, heterotrophic bacteria was carried out using tenfold serial dilutions of crude oil from the Lithuanian oilfield. The dilutions were inoculated on to Czapek agar. Inoculated agar plates were incubated aerobically at 60 °C for 48 h.

Cell morphology was examined under an Olympus AX70 microscope (magnification ×1000) and a JEM-100S electron microscope (magnification ×5000–6000) after cultivation of the strains at 60 °C on nutrient agar for 17–24 h. For bright-field microscopy, cells were stained using a Gram-staining kit (Merck). For electron microscopy, cells were prepared as described by Mignot *et al.* (2001). Bacterial size was determined by bright-field microscopy in living cell preparations from cultures grown on nutrient agar for 17–24 h. Colony morphology

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Abbreviations: ARDRA, amplified rDNA restriction analysis; RSA, ribosomal spacer analysis.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain N-3^T is AY044055.

Graphs showing the effects of temperature, pH and salinity on the growth rate of strain N-3^T are available as supplementary material in IJSEM Online.

was examined under an MBS-9 microscope (magnification $\times 4$). Colour, form, transparency, type of profile, margin and surface were recorded. Results of the morphological characterization are given in the species description.

DNA extraction and amplification of the 16S rRNA gene were performed as described by Kuisiene *et al.* (2002). The 16S rRNA gene PCR product was extracted from agarose gel using a DNA Extraction kit (Fermentas). The purified PCR product was cloned into *Escherichia coli* DH5α using the InsT/Aclone PCR Product Cloning kit (Fermentas). Recombinant clones were detected by blue/white screening (Sambrook *et al.*, 1989). Recombinant plasmid DNA was extracted as described by Birnboim & Doly (1979). The cloned 1·5 kb DNA fragments amplified by PCR were sequenced by automated DNA sequencing. The gene sequence was assembled after a minimum of 2 × sequencing coverage for each base position. 16S rRNA gene sequences were edited and the G + C mol% and sequence similarity determined using DNASTAR. The sequences were aligned with the sequence of *E. coli* (GenBank accession no. J01695; Brosius *et al.*, 1978) and nucleotides were determined in diagnostic positions (Ash *et al.*, 1993).

The 16S rRNA gene sequence determined for strain N-3^T was 1523 nucleotides long. The G + C content was 59·8 mol%. A number of nucleotides in potentially diagnostic positions (Ash *et al.*, 1993) were identified. It was established that strain N-3^T belongs to genetic group 5 of the endospore-forming bacteria.

The sequence of strain N-3^T was most similar to that of *G. thermoleovorans* DSM 5366^T, having 99·4 % sequence similarity. A search of the BLAST database also revealed the highest level of similarity with sequences of different strains of *G. thermoleovorans*. A high level of similarity was also determined for another species of genetic group 5, *B. vulcani* DSM 13174^T (99·2 % sequence similarity). Lower sequence similarities were obtained for *G. stearothermophilus* DSM 22^T, *G. thermocatenulatus* DSM 730^T, *G. kaustophilus* DSM 7263^T and *G. uzenensis* DSM 13551^T (97·4–98·4 % sequence similarity). *Geobacillus thermoglucosidasius* ATCC 43742^T and *G. caldoxylosilyticus* DSM 12041^T, as well as *G. toebii* DSM 14590^T, were the most distantly related to strain N-3^T.

The 16S rRNA gene sequences of the tested strains were aligned using the CLUSTAL_X program (Thompson *et al.*, 1997) and also manually. The size of the 16S rRNA gene used for alignment was 1415 nucleotides. A phylogenetic tree was constructed using the PHYLIP package, version 3.6a3 (Felsenstein, 2001) by the neighbour-joining method (Saitou & Nei, 1987). The evolutionary distance matrices were produced using the method of Jukes & Cantor (1969). Bootstrap analysis of the neighbour-joining data, using 1000 resamplings, was carried out to evaluate the validity and reliability of the tree topology. The tree was rooted using the X60646 sequence of *Bacillus subtilis* NCDO 1769^T as an outgroup. All analyses were carried out using the PHYLIP package version 3.6a3 (Felsenstein, 2001). Trees were visualized using TreeView software, version 1.6.1 (Page, 1996). The phylogenetic tree (Fig. 1) shows the position of strain N-3^T among the species of genetic group 5 of endospore-forming bacteria.

DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970).

Although 16S rRNA gene similarity between strain N-3^T and *G. thermoleovorans* DSM 5366^T was high, DNA–DNA relatedness was 40·0 %. DNA–DNA relatedness with *B. vulcani* DSM 13174^T was 51·0 %. *G. kaustophilus* DSM 7263^T was also chosen for DNA–DNA hybridization regarding the phylogenetic position of this species (Fig. 1). DNA–DNA relatedness with this strain was 42 %. Consequently, strain N-3^T could not be assigned to one of these three species (Vandamme *et al.*, 1996; Rosselló-Mora & Amann, 2001).

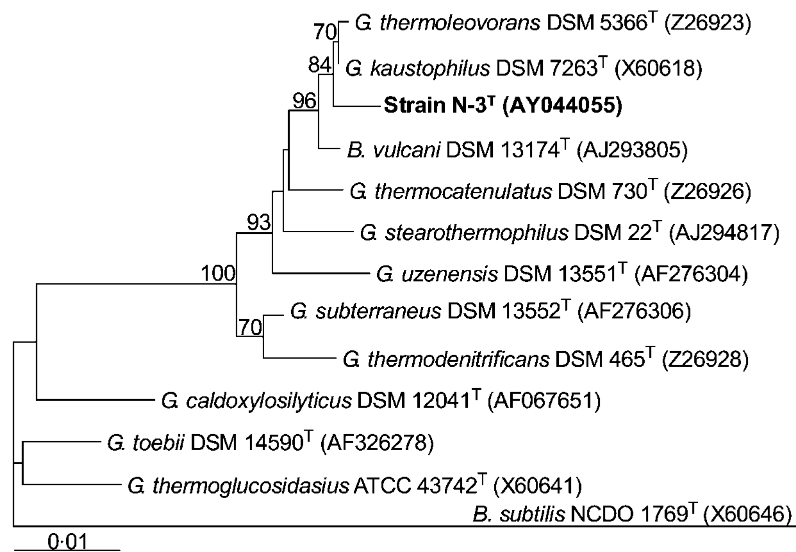

Fig. 1. Phylogenetic position of strain N-3^T among the species of genetic group 5 of the endospore-forming bacteria. The numbers at the nodes represent the percentage of bootstrap values obtained from 1000 samplings. Only the most significant values (greater than 70 %) are shown. *B. subtilis* NCDO 1769^T was defined as the outgroup of the tree. Bar, 0·01 nucleotide substitution per site.

DNA–DNA relatedness of strain N-3^T with the reference strains of the other phylogenetically related species *G. stearothermophilus* DSM 22^T, *G. thermocatenulatus* DSM 730^T and *G. uzenensis* DSM 13551^T was in the range of 32·0–52·0 %. These results showed that strain N-3^T belongs to the genus *Geobacillus*, but represents a novel species within this genus.

The G+C content of strain N-3^T was 52·5 mol%. This value is in accordance with the G+C content of the genus *Geobacillus*, which is 49·0–58·0 mol% (Nazina *et al.*, 2001).

ARDRA was performed with *Alu*I, *Hae*III and *Taq*I as described by Kuisiene *et al.* (2002). ARDRA was repeated four times using different DNA extractions for amplification and different amplification products for restriction analysis. To avoid confusion with primer dimer bands, restriction fragments shorter than 80 bp were disregarded.

The strain *G. thermoleovorans* DSM 5366^T was chosen for ARDRA as the most closely related to strain N-3^T on the basis of 16S rRNA gene analysis. *G. stearothermophilus* DSM 22^T was also included as the reference strain of the type species of the genus *Geobacillus*.

Restriction endonucleases *Alu*I, *Hae*III and *Taq*I were previously reported as suitable tools for discrimination between different species of the genus *Geobacillus* (Blanc *et al.*, 1997; Mora *et al.*, 1998; Caccamo *et al.*, 2001; Fortina *et al.*, 2001). In our study, these enzymes showed different restriction patterns for strain N-3^T and the reference strains of species *G. stearothermophilus* DSM 22^T and *G. thermoleovorans* DSM 5366^T (Fig. 2).

Strain N-3^T and *G. thermoleovorans* DSM 5366^T could be distinguished on the basis of gel-electrophoretic profiles for all three restriction enzymes tested.

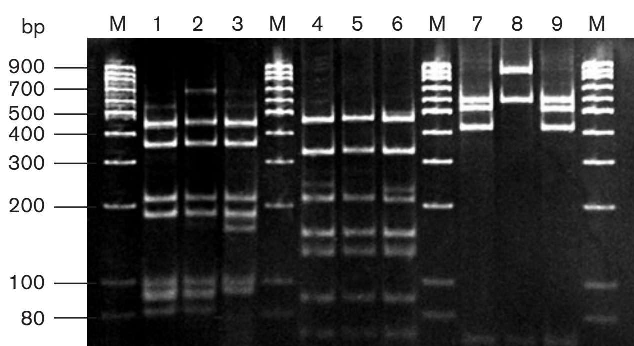

Fig. 2. ARDRA gel-electrophoretic profiles. The 16S rRNA gene was amplified by PCR using the universal bacterial primers 27F and 1495R. Lanes 1–3, *Alu*I restriction patterns; lanes 4–6, *Hae*III restriction patterns; lanes 7–9, *Taq*I restriction patterns. M, marker GeneRuler 100 bp DNA Ladder (Fermentas): 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100 and 80 bp. Lanes 1, 4 and 7, strain N-3^T; lanes 2, 5 and 8, G. *thermoleovorans* DSM 5366^T; lanes 3, 6 and 9, *G. stearothermophilus* DSM 22^T.

*Taq*I gel-electrophoretic profiles were identical for strains N-3^T and *G. stearothermophilus* DSM 22^T. Nevertheless, these two strains could be distinguished on the basis of *Alu*I analysis. A fragment of 160 bp was visible in the case of *G. stearothermophilus* DSM 22^T, while the restriction profile of strain N-3^T lacked this fragment. Instead, the *Alu*I restriction pattern of strain N-3^T had a fragment of approximately 80 bp, absent from the *G. stearothermophilus* DSM 22^T profile with this enzyme. The *Hae*III patterns of these two strains also differed, although not as markedly as in the case of *Alu*I.

In summary, our data have shown that, although strain N-3^T and the species *G. thermoleovorans* are the closest neighbours according to 16S rRNA gene analysis, they can easily be separated on the basis of ARDRA profiles.

All physiological assays were performed in duplicate and repeated three times if the obtained results were inconsistent. Unless otherwise stated, cultures were incubated aerobically at 60 °C for 24 h. Most of the physiological tests were carried out using the methods described by Claus & Berkeley (1986). Denitrification was examined as described by Blanc *et al.* (1997). Hydrolysis of collagen was tested on tap-water agar plates containing 20 g collagen l^{-1}. Resistance to streptomycin was examined on nutrient agar plates containing 10 or 50 μg streptomycin ml^{-1}. The temperature range for growth was determined as described by Manachini *et al.* (2000) and retested in nutrient broth buffered with 50 mM Tris/HCl (pH 6·5) by measuring the optical density at 600 nm. To study the influence of pH on bacterial growth, nutrient broth was buffered with citrate-phosphate buffer (pH 6·0) and 50 mM Tris/HCl (pH 6·5, 7·0, 7·5, 8·0, 8·5 and 9·0). Bacterial growth in buffered medium was monitored by measuring the optical density at 600 nm using a Beckman DU-650 spectrophotometer (results for the ranges of temperature, pH and salinity are available as supplementary material in IJSEM Online). Results of the physiological characterization are given in the species description.

Description of *Geobacillus lituanicus* sp. nov.

Geobacillus lituanicus (li.tu.a′ni.cus. M.L. adj. *lituanicus* of Lithuania, referring to the Lithuanian oilfield from where the type strain was isolated).

Cells are rod-shaped, occurring in chains, motile by means of peritrichous flagella, varying in length from 4·4 to 5·8 μm and in diameter from 1·1 to 1·4 μm. Oval subterminal endospores are produced within the slightly distended sporangia. Gram staining is positive. Colonies are small, round, tawny, convex, opaque and shiny. Obligately thermophilic, the optimal growth temperature ranges between 55 and 60 °C with a minimum at 55 °C and a maximum at 70 °C. Aerobic/facultatively anaerobic

Table 1. Differentiating phenotypic characteristics of strain N-3^T and the most phylogenetically related species of the genus *Geobacillus* and *B. vulcani*

Taxa: 1, *B. vulcani*; 2, *G. kaustophilus*; 3, *G. stearothermophilus*; 4, *G. thermocatenulatus*; 5, *G. thermoleovorans*; 6, *G. uzenensis*. +, All strains are positive; V, characteristic is variable; −, all strains are negative; ND, not determined. Data were obtained from this study or from Caccamo *et al.* (2000) (*B. vulcani*); Claus & Berkeley (1986) (*G. stearothermophilus*); Golovacheva *et al.* (1975) (*G. thermocatenulatus*); Nazina *et al.* (2001) (*G. uzenensis*); White *et al.* (1993) (*G. kaustophilus*); Zarilla & Perry (1987) (*G. thermoleovorans*). All strains were positive for utilization of glucose, fructose, maltose, mannose and sucrose.

Characteristic	Strain N-3^T	1	2	3	4	5	6
Temperature range (°C)	55–70	37–72	ND	37–65	35–78	45–70	45–65
pH	6·5	5·5–9·0	ND	ND	ND	6·2–7·5	6·2–7·8
Motility	+	+	ND	+	+	−	+
Catalase	+	−	+	V	+*	+	+
Oxidase	+	−	+	V	+*	+*	+*
Hydrolysis of:							
Casein	+	−	+	V	+	+	−
Collagen*	+	+	+	+	ND	−	−
Starch	+	+	+	+	−	V	+
Gas from nitrate	−	−	−	V	+	ND	−
Denitrification	+	−	ND	V	ND	ND	−
NaCl range (%)	0–0·5	0–3·0	ND	0–5·0	0–4·0	ND	0–4·0
Resistance to lysozyme	−	−	+	−	ND	ND	ND
Production of acid from:							
Arabinose	+	−	−	V	−	V	+
Cellobiose	+	+	+	−	+	+	+
Galactose	+	+	ND	−	+	+	+
Mannitol	+	−	ND	V	+	+	+
Ribose	+	+	−	ND	ND	+	+
Xylose	+	+	+	V	−	V	−

*Data obtained in the present study.

chemo-organotroph; nitrate is the terminal electron acceptor under anaerobic conditions. Differentiating phenotypic characteristics are indicated in Table 1. The DNA G+C content is 52·5 mol%. Isolated from the crude oil of a high-temperature oilfield.

The type strain is N-3^T (=DSM 15325^T=VKM B-2294^T).

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