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EXPERIMENTAL ARTICLES

Geobacillus uralicus, a New Species of Thermophilic Bacteria

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Abstract—The K_2^T strain of thermophilic spore-forming bacteria was isolated from a biofilm on the surface of a corroded pipeline in an extremely deep well (4680 m, 40–72°C) in the Urals. The cells are rod-shaped, motile, gram-variable. They grow on a complex medium with tryptone and yeast extract and on a synthetic medium with glucose and mineral salts without additional growth factors. The cells use a wide range of organic substances as carbon and energy sources. They exhibit a respiratory metabolism but are also capable of anaerobic growth on a nitrate-containing medium. Growth occurs within the 40–75°C temperature range (with an optimum of 65°C) and at pH 5–9. The minimum generation time (15 min) was observed at pH 7.5. Ammonium salts, nitrates, and arginine are used as nitrogen sources. The G+C content of the DNA is 54.5 mol %. From the morphological, physiological, and biochemical properties and the nucleotide sequence of the 16S rRNA gene, it was concluded that the isolate K_2^T represents a new species of the genus *Geobacillus, Geobacillus uralicus.*

Key words: thermophilic bacteria, *Geobacillus uralicus*, taxonomy.

The genus *Geobacillus* was described in 2001 by Nazina *et al.* [1] to include the new isolates *G. subterraneus* and *G. uzenensis* and the bacteria *G. stearothermophilus, G. thermoleovorans, G. thermocatenulatus, G. kaustophilus, G. thermoglucosidasius*, and *G. thermodenitrificans* that had been previously assigned to the genus *Bacillus.*

The representatives of the genus *Geobacillus* are gram-positive spore-forming chemoorganotrophs characterized by aerobic or facultatively anaerobic metabolism. All geobacilli are obligatory thermophiles. The type species is *Geobacillus stearothermophilus* DSM 22^T [1].

The goal of this work was to investigate the morphological, physiological, and biochemical properties and to determine the taxonomic position of the thermophilic strain K_2^T isolated in culture from a biofilm on a corroded pipeline in a high-temperature well.

MATERIALS AND METHODS

Isolation source and cultivation conditions. The thermophilic bacterium was isolated from an enrichment culture at 60°C in a mineral medium containing (g/l) K_2HPO_4, 0.05; KH_2PO_4, 0.05; $MgSO_4$, 0.04; NaCl, 0.1; $CaCl_2$, 0.01; $(NH_4)_2SO_4$, 0.5; and glucose, 10. Cultivation was performed without agitation of the medium. For inoculation, we used a sample of a corroded pipeline (1 × 1 × 1 cm) from an extremely deep well (SG-4) of the Ural Geological Prospecting Expedition. The temperature in the pipeline and in the well was 40–72°C. The pure culture was obtained by plating the enrichment culture onto nutrient agar (Dagestan Research Institute for Nutrient Media) with subsequent subculturing.

The microorganisms were maintained on agarized LB medium [2]. Comparative studies additionally used strains *G. stearothermophilus* DSM 22^T and *G. thermoleovorans* DSM 5366^T and *G. uzenensis* strains U^T and X and *G. subterraneus* strain 34^T from the microbial culture collection of the Laboratory of Oil Microbiology (these strains were kindly provided by T.N. Nazina).

Microscopy. Morphological examinations were conducted with preparations of living and fixed (gram-stained) bacteria, using a Docuval microscope (Germany) equipped with a phase-contrast device.

Biochemical tests and analytical methods. The physiological and biochemical properties of the bacteria were studied by conventional methods [3–5].

The utilization of various compounds as carbon and energy sources by the bacteria was tested on a mineral

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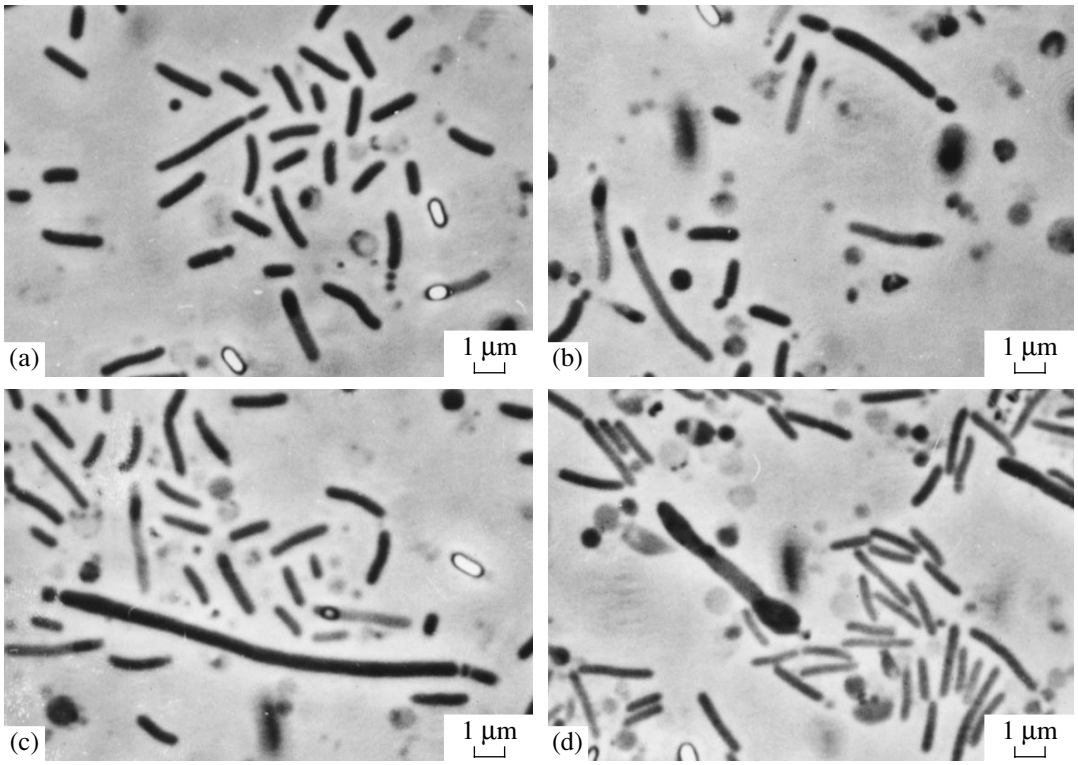

Fig. 1. Cell morphology of strain K_2^T: (a) in the growth retardation phase (a 5-h culture); (b–d) in the stationary phase (a 24-h culture).

medium [6]. The substrates were sterilized separately and added to the medium at a concentration of 1%, except for hydrocarbons, which were added at concentrations of 0.1–1%.

The utilization of various carbon sources was judged from the increase in light scattering of the liquid medium in three consecutive subcultures. The utilization of various nitrogen sources was monitored in the same mineral medium that was supplemented with potassium nitrate, ammonium sulfate, or arginine in amounts that corresponded to equivalent nitrogen doses. The capacity to grow at various values of medium salinity was tested in mineral medium with glucose.

The temperature and pH ranges for the growth of bacteria were determined using kinetic criteria: the specific growth rates of bacteria were determined on the mineral medium [6] and on a complex medium containing 2.5 g/l yeast extract and 5 g/l tryptone (pH 7.0).

Dissociant forms were isolated from the population by plating a microbial suspension onto agar medium composed of equal amounts of wort and LB medium.

Determination of the fatty-acid composition. To determine the fatty-acid composition of bacteria, we used biomass grown on LB medium at 60°C for 18 h.

A biomass sample was dehydrated by passing inert gas over it at 80°C and treated with 0.4 ml of a 1 N solution of hydrogen chloride in methanol at 80°C for 3 h (acidic methanolysis). The methyl esters of fatty acids resulting from methanolysis and other lipid components were extracted with hexane. The hexane was evaporated, and the dry substances were silylated with 20 μl bis-(trimethylsilyl)-trifluoroacetamide for 15 min at 80°C and diluted with hexane to a volume of 100 ml. A 1-μl aliquot of the mixture was injected into an HP-5973 Hewlett Packard gas chromatograph–mass spectrometer (United States) operating in an automatic mode. The quadrupole mass spectrometer used electrons with an energy of 70 eV for ionization and had a resolution of 0.5 amu and a working mass range of 2–1000 amu; the sensitivity threshold was 0.01 ng of methyl stearate. The chromatograph was equipped with a 25-m-long molten quartz capillary column with an inner diameter of 0.25 mm. HP-5 ms (Hewlett Packard) with a layer thickness of 0.2 μm served as the stationary phase. The temperature was programmed to increase from 120 to 280°C at a rate of 5°C/min during chromatography. The temperature of the injector and the interface was 280°C. The data were processed using the stock software of the device. Sample components were identified based on the known features of their mass spectra and relative retention times, using the Willey275 and NBS75k spectral libraries and the ChemStation software (Hewlett Packard).

DNA analysis. DNA was isolated by the Marmur method [7]. The nucleotide composition of DNA was determined from its melting temperature on a Pye Uni-

cum SP1800 self-recording spectrophotometer operated at a heating rate of 0.5°C/min. The G+C content was calculated using the formula G+C = $2.08T_{melt}$ – 106.4 mol %. The DNA of *Escherichia coli* K-12 was employed as a standard. The DNA–DNA homology levels were determined from the reassociation rates using the method of De Ley *et al.* [9].

Amplification, sequencing, and phylogenetic analysis of the 16S rRNA gene. The 16S rRNA gene was amplified and sequenced employing universal primers applicable to most prokaryotes [10]. Buffer containing 1.5 μM $MgCl_2$, 50 mM KCl and 10 mM Tris–HCl (pH 8.3) and 0.001% gelatine were used for amplification. The volume of the reaction mixture was 100 μl; it contained standard dNTP concentrations and equimolar amounts of the pA and pH' primers. 30 amplification cycles were run, using the following temperature regime: DNA denaturation at 94°C for 30 s, primer annealing at 40°C for 1 min, and elongation at 72°C for 2 min 30 s. Following purification on low-jelling-temperature agarose and Promega columns, the 16S rRNA gene was sequenced in both directions using forward and reverse universal primers and the sequenase enzyme (Biochemicals, Cleveland, Ohio, USA).

The results of sequencing of the complete 16S rRNA gene of strain K_2^T were deposited with the GenBank (accession number, AY 079151).

The nucleotide sequence of the 16S rRNA gene of strain K_2^T was manually aligned with the corresponding sequences of the species of the genus *Geobacillus*. The sequences containing unidentified nucleotides were disregarded, and, as a result, 1405 nucleotides were compared. The phylogenetic tree of the tested bacteria was constructed using the methods implicated in the TREECON software package [11].

RESULTS AND DISCUSSION

Morphological and colonial features. The cells of strain K_2^T exhibited gram-variable staining (a 18-h culture stained gram-negative, whereas older cultures stained gram-positive). The colonies on LB medium were rounded (2–4 mm in diameter), smooth, convex, even-edged, transparent, colorless, structurally homogeneous, viscous. The rod-shaped cells contained in a colony were either solitary or arranged in pairs; only seldom did they form chains. On LB medium with wort, colonies of two types were formed: (i) rounded smooth convex colonies with even edges (1–2 mm in diameter) and (ii) larger (2–5 mm in diameter) wrinkled colonies with wavy edges. Probably, the culture dissociated into the S and R forms. The dissociant ratio in the population changed depending on the culture age. A 100% content of the S forms occurred in the late exponential and early stationary phase, and the R forms tended to dominate the late-stationary-phase culture (only 20% of the cells were still in the S form). Of considerable importance was also the pH of the medium. The R forms prevailed at low pH values, and the S forms prevailed in neutral and alkaline media.

Table 1. Fatty-acid composition of *G. uralicus*

Fatty acid	% of total fatty acids
12 : 0 lauric	1
14 : 0 myristic	3
*i*15 : 0 isopentadecanoic	24.3
*a*15 : 0 anteisopentadecanoic	0.4
15 : 0 pentadecanoic	1.1
*i*16 : 0 isopalmitic	3.5
16 : 1 9,10-hexadecenoic	2.2
16 : 0 palmitic	23.3
*i*17 : 0 isoheptadecanoic	16.6
*a*17 : 0 anteisoheptadecanoic	5.8
17 : 0 heptadecanoic	3.2
18 : 1^9 oleic	6.1
18 : 0 stearic	8.3

The vegetative cells of strain K_2^T were slightly curved motile peritrichously flagellated rods occurring either singly or in pairs (Fig. 1). Forespores, sporulating cells, and free spores appeared at the end of the growth retardation phase. The ellipsoidal spores occupied a terminal position in the maternal cells and somewhat expanded them (Fig. 1a). The cell size was 0.6–1.2 × 2.4–8.8 μm. Cells of a larger size (up to 30 μm in length) also occurred, which was presumably due to impaired cell division. It should be noted that strain K_2^T cells displayed pleomorphism, a characteristic feature of thermophiles. In addition to large cells, the culture contained mini cells located at the poles of "normal" cells (which could harbor 1 to 2 such mini cells), cells with bulges, and spherical cells (Fig. 1).

Nutritional requirements and physiological properties. Strain K_2^T is an obligate thermophile. It is capable of utilizing a wide range of carbohydrates, alcohols, polycarboxylic acids, and hydrocarbons (Table 1). Under microaerobic conditions (in tubes half-filled with liquid medium and stoppered with cotton plugs), it reduced nitrates to nitrites. It grew in liquid LB medium containing 0.1% potassium nitrate under anaerobic conditions [3], on agarized Ashby medium, and in liquid nitrogen-free medium. The strain could also use potassium nitrate, ammonium sulfate, and arginine as the nitrogen sources. It failed to grow autotrophically on an H_2 + CO_2 mixture. Strain K_2^T grew on synthetic medium without vitamins, NaCl, and KCl; its growth also occurred on nutrient-depleted agar. The strain showed good growth on LB medium. Strain

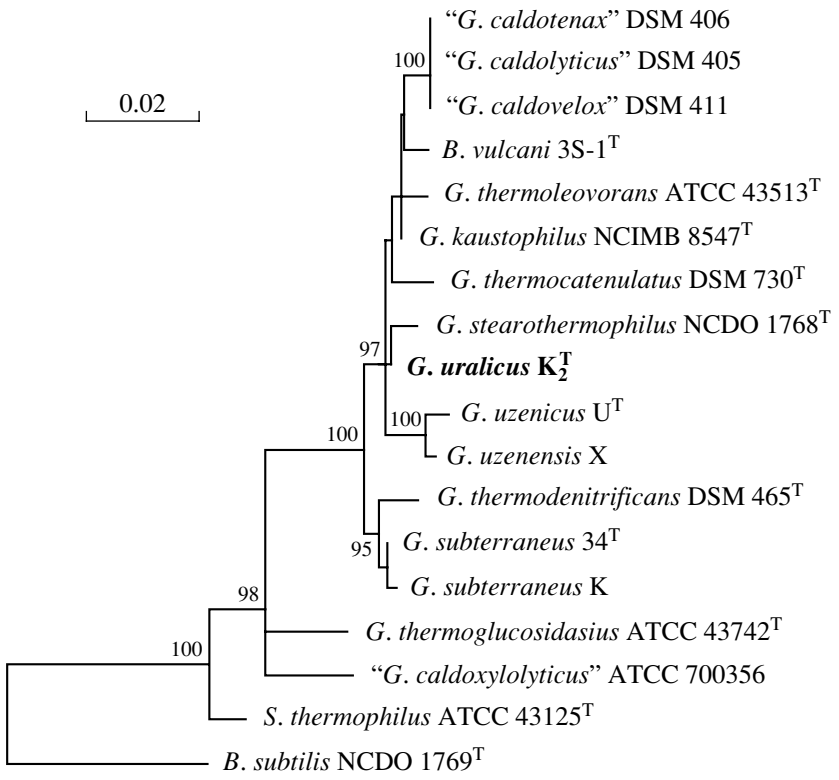

Fig. 2. Phylogenetic tree of the genus *Geobacillus* based on comparative studies on the 16S rDNA sequences. The position of strain K_2^T is shown in boldface. Scale bar corresponds to 5 nucleotide substitutions per 100 nucleotides. The numerals show data of bootstrap analysis of the significance levels of the branching order (bootstrap values above 95 are considered significant).

K_2^T formed acid but not gas from galactose, ribose, glycerol, xylose, glucose, fructose, maltose, mannitol, mannose, and sucrose; it formed no acid from arabinose, lactose, rhamnose, sorbitol, sorbose, or raffinose. It used peptone, tryptone, yeast extract, succinate, pyruvate, formate, acetate, lactate, butyrate, benzoate, citrate, methanol, ethanol, phenol, and tetradecane. Strain K_2^T did not ferment glucose; it was catalase- and oxidase-positive. It did not form NH_3 on nutrient broth. The strain failed to deaminate phenylalanine, could not degrade urea and tyrosine, and did not evolve H_2S. The result of the methyl red test was negative. Strain K_2^T did not form indole, dihydroxyacetone, or acetoine (the Voges–Proskauer test yielded negative results).

Table 2. G+C content of the DNA and the homology level between *G. uralicus* and some representatives of the genus *Geobacillus*

Strain	G+C content, mol %	DNA homology level, %
G. uralicus K_2^T	54.5	100
G. uzenensis (strain X)	53.0	35
G. uzenensis (strain U^T)	51.3	40
G. subterraneus (strain 34^T)	52.3	41
G. thermoleovorans DSM 5366^T	53.7	53
G. stearothermophilus DSM 22^T	55.2	37
Escherichia coli K-12	51.7	–

The highest growth rate on a rich medium was observed at 65°C and pH 7.5. On mineral medium, the pH optimum was the same, but the temperature optimum dropped to 60°C, and the growth-compatible temperature range narrowed. The specific growth rate was 2.7 and 1.2 h^{-1} on a complex and synthetic medium, respectively. Thus, our isolate grew within the temperature and the pH ranges characteristic of its habitat. Importantly, this microorganism consumed hydrocarbons, lower molecular weight alcohols, and volatile fatty acids, which, as a rule, occur in subterranean environments [12]. The addition of NaCl in an amount as low as 0.5% caused a certain decrease in the specific growth rate on mineral medium.

The fatty acid spectrum of strain K_2^T lacked hydroxy acids (Table 2), which is typical of gram-positive bacteria. Branched fatty acids prevailed in this strain. Iso-

Table 3. Physiological and biochemical properties of *G. uralicus* and some other representatives of the genus *Geobacillus*

Character	*G. uralicus*	*G. usenensis*		*G. subterraneus*	*G. stearothermophilus*	*G. thermoleovorans*	*G. kaustophilus*
		Strain U^T	Strain X	Strain 34^T			
Cell width, mm	0.6–1.2	0.9–1.3	1.0–1.7	0.8–1.2	0.6–1	0.9	1.5
Cell length, μm	2.4–8.8	4.7–8.0	5.5–8.5	4.6–6.6	2–3.5	6–8	3.5
Motility	+	+	+	+	+	+	–
Catalase	+	+	+	+	+	+	ND
NH_3 formation on nutrient broth	–	+	+	+	ND	ND	ND
Phenylalanine deamination	–	–	–	–	ND	ND	ND
Urea degradation	–	–	–	–	ND	ND	ND
H_2S formation	–	–	–	–	ND	ND	ND
Indole formation	–	–	–	–	ND	ND	ND
Lecithinase test with egg yolk	–	–	–	–	ND	ND	ND
Voges–Proskauer test	–	–	–	–	ND	–	ND
Acid (not gas) formation from							
adotinol	–	–	–	–	ND	ND	ND
L-arabinose	–	+	+	–	D	–	D
galactose	+	+	+	+	–	+	+
ribose	+	+	+	+	ND	ND	+
glycerol	+	+	+	+	+	+	D
inositol	+	–	–	–	–	–	–
lactose	–	–	–	–	–	–	–
rhamnose	–	–	–	–	–	–	–
sorbitol	–	–	–	–	–	ND	–
D-xylose	+	–	–	–	D	–	D
glucose	+	+	+	+	+	+	ND
fructose	+	+	+	+	+	+	ND
maltose	+	+	+	+	+	+	ND
mannitol	+	ND	ND	ND	ND	ND	ND
mannose	+	+	+	+	+	+	ND
sucrose	+	+	+	+	+	+	ND
sorbose	–	ND	ND	ND	ND	–	ND
raffinose	–	–	–	–	–	–	ND
Gelatin hydrolysis	+	+	+	–	D	–	ND
Casein hydrolysis	+	–	–	–	D	ND	+
Starch hydrolysis	+	+	+	+	+	–	D
Esculin hydrolysis	+	+	+	+	ND	ND	ND
Utilization of							
n-alkanes	+	+	+	+	+	+	+
formate	+	–	–	+	–	ND	ND
acetate	+	+	+	+	+	ND	+
lactate	+	+	+	+	–	ND	ND
butyrate	+	+	+	+	+	+	ND
benzoate	+	+	+	+	+	+	ND
ethanol	+	+	+	+	+	+	ND
phenol	+	+	+	+	+	+	ND
Simons citrate	+	–	–	–	D	+	ND
pyruvate	+	+	+	+	+	+	ND
succinate	+	+	+	+	+	+	ND
Glucose fermentation	–	–	–	–	D	+	–
Denitrification	+	–	–	+	–	+	ND
Methyl red test	–	–	–	+	D	ND	ND
NaCl concentration range enabling growth, %	0–3	0–4	0–4	0–3	0–5	0–4	ND
pH range enabling growth	5–9	6.2–7.8	6.2–7.8	6.2–7.8	6.0–8.0	6.2–7.8	6.2–7.5
Temperature range enabling growth, °C	40–75	45–65	45–65	45–65	37–65	35–78	40–75
G+C content, mol %	54.5	50.4	51.5	52.3	519	552	52–78
Reference	Our data	[1]	[1]	[1]	[1]	[1]	[1]

Note: "+," the character is positive; "–," the character is negative; D, 11–89% of the strains yield positive results; ND, no data.

pentadecanoic, palmitic, and isoheptadecanoic acids were the predominant species. The prevalence of isopentadecanoic and isoheptadecanoic acids had been earlier established in other representatives of the genus *Geobacillus*. Anteisopentadecanoic (in line with the data available in the literature [1]), lauric, and pentadecanoic acids also occurred in small amounts.

Genotype characterization. The G+C content in the DNA of strain K_2^T was 54.5 mol %, a value close to those reported for the species of the genus *Geobacillus*. The genomic similarity level between strain K_2^T and *Geobacillus* representatives was 35–53%, as determined in DNA reassociation studies (Table 2). Such a genomic similarity level testifies to the affiliation of our isolate to the genus *Geobacillus* [13], but it does not suffice for classifying strain K_2^T into one of the *Geobacillus* species described earlier [14].

Phylogenetic analysis. We determined the nucleotide sequence of a large portion of the 16S rRNA gene (1426 nucleotides corresponding to positions 39–1449 in terms of the *E. coli* numbering). A comparative analysis of the nucleotide sequence of the 16S rRNA gene revealed (Fig. 2) that strain K_2^T belongs to the cluster of species of the genus *Geobacillus* (96.4–99.6% homology). Among the *Geobacillus* species, strain K_2^T is most close to *G. kaustophilus* (99.6%) and *G. stearothermophilus* (99.5%).

Taxonomic position. From our studies, we conclude that strain K_2^T should be classified with the genus *Geobacillus* [1] based on its morphological, physiological, and biochemical features (Table 3). It should be noted that representatives of this genus (*G. thermocatenulatus*, *G. stearothermophilus*, and *G. thermodenitrificans*) were earlier isolated from a similar habitat: mucilaginous film on the inner surface of a pipe in a high-temperature well (60–65°C) in the thermal zone of the Yangan-Tau Mountain (southern Ural) [15, 16].

Based on the phylogenetic analysis of the 16S rRNA genes, strain K_2^T belongs to the genus *Geobacillus*. This is consistent with the high degree of similarity (35% or above) between its total DNA and the DNA of other representatives of this genus. Although strain K_2^T is very close to the type species *G. stearothermophilus* in the nucleotide sequence of its 16S rRNA gene, it cannot be classified with this species because of the insufficient DNA–DNA hybridization level (37%) and some phenotypic differences (*G. uralicus* and *G. stearothermophilus* differ in their cell size, temperature and salinity growth ranges, capacity to consume L-arabinose, galactose, inositol, formate, and lactate, and the results of the methyl red test). The 16S rRNA similarity level between most of the known species of the genus *Geobacillus* is high (98.0–99.6%), and they are differentiated using DNA–DNA hybridization data and phenotypic analysis [1]. The DNA–DNA hybridization level between the new strain and the reference strains used in comparative studies did not exceed 53% and was maximum with *G. thermoleovorans*. As distinct from this species, strain K_2^T forms acid but not gas from inositol and D-xylose and hydrolyzes gelatine and starch (Table 3).

Hence, based on our studies using the methods of polyphasic taxonomy, strain K_2^T can be considered to represent a new species of the genus *Geobacillus*.

Species description. *Geobacillus uralicus* sp. nov. (u.ra'li.cus, from the non-Latin adjective *uralicus*, related to the Urals). The species name reflects the geographical location of the isolation source: surface biofilm in a pipe from a well situated in the Urals.

Curved rods (0.6–1.2 × 2.4–8.8 μm), single or arranged in pairs, gram-variable, motile, peritrichously flagellated. Ellipsoid spores locate terminally in the maternal cells and somewhat expand them. Colonies on agarized LB medium are rounded, 2–4 mm in diameter, smooth, convex, even-edged, transparent, structurally homogeneous, viscous. Growth occurs within the temperature range of 40– 75°C at pH 5–9 with an optimum at 65°C and pH 7.5. Chemoorganotrophic, aerobic or facultatively anaerobic. Oxygen or nitrate are used as terminal electron acceptors. Growth factors, vitamins, NaCl or KCl are not required.

Acid, but not gas, is formed from galactose, ribose, glycerol, inositol, xylose, glucose, fructose, maltose, mannitol, mannose, and sucrose, but not from adonitol, arabinose, lactose, rhamnose, sorbitol, sorbose, or raffinose. Alkanes, formate, acetate, lactate, butyrate, benzoate, methanol, ethanol, phenol, citrate, pyruvate, and succinate are utilized as carbon and energy sources. Catalase activity is present. Phenylalanine is not deaminated. Urea and tyrosine are not degraded. Indole, dihydroxyacetone, ammonia, and hydrogen sulfide are not formed. The Voges–Proskauer, methyl orange, and lecithinase tests yield negative results. Ammonium salts, nitrates, and amino acids are used as nitrogen sources. Growth is possible on Ashby medium and in liquid nitrogen-free mineral medium. Autotrophic growth does not occur. Starch, gelatin, casein, and esculin are hydrolyzed.

Isopentadecanoic, palmitic, and isoheptadecanoic acid are the predominant fatty acids.

The G+C content of the DNA is 54.5 mol %.

The type strain *Geobacillus uralicus* K_2^T was isolated from a surface biofilm on a pipe and deposited with DSMZ (no. 14577) and VKM (no. 2276).

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