***Anoxybacillus pushchinensis* gen. nov., sp. nov., a novel anaerobic, alkaliphilic, moderately thermophilic bacterium from manure, and description of *Anoxybacillus flavithermus* comb. nov.**

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A new strictly anaerobic, alkaliphilic, moderately thermophilic, fermentative, spore-forming bacterium, strain K1^T, was isolated from manure samples (pH 6–8). Cells were Gram-positive, straight, non-motile rods that grew at temperatures of 37–66 °C (optimum at 62 °C) and in a pH range of 8.0–10.5 (optimum at 9.5–9.7). The bacterium fermented D-glucose, sucrose, D-fructose, D-trehalose and starch as carbon and energy sources. It required vitamins and its growth is stimulated by yeast extract. The major metabolic products were H₂ and acetate. Cells were catalase-negative and could reduce nitrate to nitrite. The G+C content of the DNA was 42.2 mol%. Based on the phenotypic properties and 16S rDNA sequencing and DNA–DNA hybridization data, strain K1^T (= DSM 12423^T = ATCC 700785^T = VKM B-2193^T) was assigned to the new genus *Anoxybacillus* gen. nov., as a representative of a new species, *Anoxybacillus pushchinensis* sp. nov. ‘*Bacillus flavothermus*’ strain d.y., which was found to be closely related to strain K1^T, is described as *Anoxybacillus flavithermus* comb. nov. (type strain = d.y.^T = DSM 2641^T).

Keywords: anaerobic alkaliphilic thermophilic bacterium, *Anoxybacillus pushchinensis* gen. nov., sp. nov., *Anoxybacillus flavithermus* comb. nov.

INTRODUCTION

Alkaliphilic bacteria can be subdivided into two groups which include obligately alkaliphilic species that are unable to grow at neutral pH, and alkali-tolerant bacteria that are able to grow at high pH values but have their growth optimum at or near a neutral pH (Grant & Tindall, 1986). Alkalitolerant bacteria of the species *Streptococcus faecalis* were first described in 1928 (Downie & Cruickshank, 1928). Obligately alkaliphilic bacteria of the genera *Bacillus* and *Clostridium* have been isolated from soils with neutral pH (Horikoshi & Akiba, 1982). Later, meso-

philic alkaliphilic bacteria were found in soda lakes (Grant & Tindall, 1986; Krulwich & Guffanti, 1989; Zavarzin, 1993). More recently, thermophilic, alkaliphilic, anaerobic bacteria have been isolated from sewage (Li *et al.*, 1993, 1994) and thermal springs (Engle *et al.*, 1995). These investigations expanded our knowledge about the biodiversity and ecology of alkaliphilic bacteria. For some of these bacteria, however, the optimal growth conditions differ remarkably from the conditions in their habitats and the role of such micro-organisms in these ecosystems is not well understood yet. In this work, *Anoxybacillus pushchinensis* gen. nov., sp. nov. is described, a new species of the strictly anaerobic, moderately thermophilic, obligately alkaliphilic bacilli isolated from manure with neutral pH. Since the new species was found to be closely related genetically to ‘*Bacillus flavothermus*’ (Heinen *et al.*, 1982), this hitherto

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The GenBank accession number for the 16S rDNA sequence of *Anoxybacillus pushchinensis* strain K1^T is AJ010478.

invalidly named species is described as *Anoxybacillus flavithermus* comb. nov. (name corrected).

METHODS

Strains, media and culture conditions. Strain K1^T was isolated from enrichment cultures that had been inoculated with mixed manure samples and grown under anaerobic conditions in Hungate tubes at 60 °C for 4 d. Samples of equine and porcine manure (1:1, w/w) with a pH of 6.0–7.0 were collected from farms in the Moscow region and stored at 4 °C before inoculation.

For the enrichment, isolation and cultivation of strain K1^T, a previously described anaerobic basal medium (Zhilina *et al.*, 1997) was used with certain modifications. The medium contained (g l⁻¹): KH₂PO₄, 0.2; MgCl₂·6H₂O, 0.1; KCl, 0.2; NH₄Cl, 1.0; Na₂CO₃, 2.76; NaHCO₃, 10.0; NaCl, 5.0; Na₂S·9H₂O, 0.5. The pH of the medium after autoclaving was 9.5–9.7. After sterilization at 120 °C for 30 min, yeast extract (0.01 g l⁻¹) and glucose as carbon source (5.0 g l⁻¹) were added. Medium (1 l) was supplemented with 10 ml of a stock vitamin solution (Wolin *et al.*, 1963) and 1 ml of a trace element solution containing (mg per 200 ml water): MnCl₂·4H₂O, 720; Fe(NH₄)(SO₄)₂·12H₂O, 400; FeSO₄·7H₂O, 200; CoCl₂·6H₂O, 200; ZnSO₄·7H₂O, 200; Na₂MoO₄·2H₂O, 20; NiCl₂, 100; CuSO₄·5H₂O, 20; AlK(SO₄)₂·12H₂O, 20; H₃BO₃, 20; and 5 ml concentrated HCl.

Unless otherwise indicated, enrichment and pure cultures were grown at 55–62 °C in 15 ml Hungate tubes containing 10 ml medium and filled with N₂. All sampling and dispensing procedures were performed with sterile syringes and needles. Prior to roll-tube cultivation on 3% Difco agar (w/v), aliquots of separately sterilized carbonate solutions were added, in addition to yeast extract and glucose, to Hungate tubes with 4 ml sterile basal medium.

In the series of experiments designed to elucidate the optimal growth conditions, the pH of the medium was adjusted to required values with sterile stock solutions of HCl or NaOH at 55 °C under an N₂ flow. pH measurements were done using a model 121 pH meter (Russia) calibrated at 55 °C. Cells were grown at 55 °C. To determine the range of growth temperatures, the isolate was cultivated in the basal medium (pH 9.7).

The effect of NaCl on the bacterial growth was studied in basal medium containing 0.0, 0.5, 1.0, 2.0, 3.0, 5.0 or 7.0% (w/v) NaCl. The medium was modified to exclude other sodium sources by replacing Na₂CO₃ and NaHCO₃ with 5 g l⁻¹ K₂CO₃ and Na₂S with K₂S. The NH₄Cl-dependence of the strain was estimated in medium containing 0, 0.5, 1.0 or 3.0% (w/v) NH₄Cl.

Carbon sources other than glucose were added to the medium in the form of autoclaved or filter-sterilized solutions to a final concentration of 5 g l⁻¹. Sterile stock solutions of electron acceptors were added to the medium with glucose (5 g l⁻¹) to the following final concentrations: Na₂SO₄, 20 mM; Na₂SO₃, 5 mM; Na₂S₂O₃·5H₂O, 10 mM; NaNO₃, 10 mM; benzenesulfonate, 5 mM; fumarate, 10 mM; S⁰, 2 g l⁻¹.

Light and electron microscopy. The morphology of the isolate was examined under an MBI-3 LOMO (Russia) microscope with a phase-contrast device. For electron microscopy studies, harvested cells were pre-fixed with 1.5% (v/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 1 h at 5 °C, washed three times with the buffer, and fixed

with 1% OsO₄ in 0.05 M cacodylate buffer (pH 7.2) for 4 h at 20 °C. After dehydration in a series of ethanol solutions, cells were embedded in Epon 812 and sectioned on a microtome, LKB 2128 Ultratome. Ultrathin sections were placed onto copper grids and negatively stained with 3% uranyl acetate in ethanol with lead citrate (Reynolds, 1963). Thin sections were examined under a JEOL JEM-100B electron microscope.

Analytical, physiological and biochemical methods. The bacterial cell number was determined by direct counting under a light microscope. The optical density (OD) of cell suspensions was measured in 1 cm cuvettes at 600 nm using a model 11 Specol spectrophotometer. Catalase activity was assessed as described elsewhere (Gerhardt *et al.*, 1984) with 3% H₂O₂ added to the culture liquid. Volatile products of glucose fermentation were analysed with the use of a Pye Unicam 304 GC with FID and a 0.9 m × 3.3 mm Chromosorb 101 column kept at 160 °C; CO₂ served as the carrier gas. NO₂ was determined by the colorimetric method (Tiedje, 1982).

Cellular fatty acids. Fatty acids and other lipid components were extracted from cell biomass by acid methanolysis. Wet biomass (30 mg) was dried in a stream of nitrogen and 400 ml of a 1 M solution anhydrous HCl in methanol was added. The mixture was heated at 80 °C for 3 h. The methyl esters of fatty acids and dimethylacetates (aldehyde derivatives) were obtained as a result, and were extracted twice with 200 ml hexane. The extract was evaporated to dryness and silylated in 20 ml *N,O*-bis(trimethylsilyl)trifluoroacetamide for 15 min at 80 °C. A 1 ml portion of the reaction mixture was analysed with a model QP-2000 GC-MS system (Shimadzu) equipped with a fused silica capillary column (25 m × 0.25 mm) containing an Ultra-1 non-polar methyl-silicone phase. The temperature profile included a 2 min isotherm at 120 °C and subsequent temperature programming at a rate of 5 °C min⁻¹ to 280 °C. Data processing was carried out with standard programs of the GC-MS system.

Resistance to antibiotics. Resistance to antibiotics was evaluated by growing the bacterium in basal medium supplemented with filter-sterilized bacitracin, chloramphenicol (100 µg ml⁻¹), penicillin, ampicillin, streptomycin or vancomycin (all at 250 µg ml⁻¹). After the inoculation of antibiotic-containing media with exponentially growing cells, the cultures were incubated at 37 °C for 12 h and then at 55 °C for 14 d.

G + C content determination and DNA hybridization. Cells were disrupted with a French pressure cell and DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977). The G + C content of the DNA was determined by HPLC as described by Tamaoka & Komagata (1984) and Mesbah *et al.* (1989). Non-methylated lambda phage DNA (Sigma) with a G + C content of 49.858 mol% served as a standard. DNA–DNA hybridization was performed as described by De Ley *et al.* (1970) with modifications described by Huß *et al.* (1983) and Escara & Hutton (1980), using a Gilford System 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. The renaturation rates were computed using the TRANSFER.BAS program (Jahnke, 1992).

Phylogenetic analysis. Chromosomal DNA of strain K1^T was isolated and purified as described elsewhere (Marmur, 1961) and 16S rDNA was then amplified by *Thermus aquaticus* thermostable DNA polymerase (USB) via PCR in a mixture containing 1 × TaqPol buffer (USB), 0.2 mg chromosomal DNA, 20 pM oligonucleotide primers pA and pH' (Edwards *et al.*, 1989), 2.5 mM dNTP, 2.5 mM MgCl₂

and 2 U Taq. After denaturation at 94 °C for 5 min, the reaction mixture was subjected to 30 thermal cycles (52 °C, 1 min; 72 °C, 1.5 min; 94 °C, 1 min). PCR products were sequenced using the FemtoMol kit (Promega). 16S rDNA was sequenced in both strands by Sanger's method using next primers: pA, pC, pE, pD', pF' and pH' (Edwards *et al.*, 1989). All procedures were done as directed in the manufacturer's protocol. The 16S rDNA sequence of strain K1^T was aligned against those of closely related strains using the CLUSTAL program (version 1.60). Pairwise evolutionary distances were computed by the correction of Jukes and Cantor (Felsenstein, 1989) using the DNADIST program from the PHYLIP software package (version 3.5). A 16S rDNA sequence of 1340 nt was registered in GenBank under the accession number AJ010478.

RESULTS

Enrichment and pure cultures

A population of anaerobic, alkaliphilic, moderately thermophilic bacteria developed in glucose-containing medium (pH 10.0) that had been inoculated with mixed manure samples, which had a neutral pH (6.8), and incubated under anaerobic conditions at 60 °C for 4 d.

From this enrichment culture (OD of 0.4), serial dilutions were prepared and used for inoculation of the same medium supplemented with penicillin (500 µg ml⁻¹) and streptomycin (500 µg ml⁻¹). The highest dilution exhibiting growth of anaerobic alkaliphilic bacteria was pure as judged from the absence of growth at pH 7.0 in glucose-peptone medium with large amounts of yeast extract. This culture was anaerobically grown in roll-tubes with agar medium, and one of the colonies formed (referred to as strain K1^T) was selected for further studies.

Morphology

Strain K1^T was a Gram-positive, non-motile, straight, spore-forming rod with rounded ends, measuring 0.4–0.5 × 2.5–3.0 µm. It formed terminal and sub-terminal spherical endospores. Interestingly, spores were found only after storage at –40 °C and 12 h post-incubation at 22 °C. Cells occurred singly, in pairs or in short or, sometimes, long chains (Fig. 1a). Cells reproduced by binary fission; Y-shaped cells sometimes occurred (Fig. 1b, f). The cell wall, visible as a narrow electron-dense layer, was of the Gram-positive type. The outer part of the cell envelope consisted of two structurally ordered S-layers covered with a thick peptidoglycan layer (Fig. 1d).

Cultural, physiological and biochemical properties

After 2–3 d cultivation on agar medium at 55 °C, the colonies of strain K1^T were white, matt, dentate, rough and from 0.5 to 2.0 mm in diameter. Surface colonies were round and granular, with a dense centre. Sub-surface colonies were round, white, with a dense yellowish centre and uneven edges.

Strain K1^T grew strictly anaerobically ($E_h < -100$

mV) in medium with glucose as the carbon and energy source; growth was stimulated by vitamins and yeast extract. The bacterium was catalase-negative and able to reduce nitrate to nitrite. It did not hydrolyse gelatin or casein.

Growth was observed in a pH range of 8.0–10.5 (Fig. 2a), with an optimum at 9.5–9.7. At pH 9.7, cells began to multiply after a very short lag phase. Strain K1^T could not grow in media with pH values of 7.5 or 11.0. The bacterium obligately required carbonate for growth; it could not grow in carbonate-free glycine- or serine-buffered media (pH 9.5) with glucose (5 g l⁻¹). Strain K1^T grew well, however, in carbonate-containing medium with glycine and serine (5 g l⁻¹); therefore, these amino acids had no inhibitory effect. Hence, strain K1^T is an alkaliphile that is obligately dependent on carbonate. The concentrations of NaCl optimal for growth ranged from 0.5 to 1 % (w/v). The strain was tolerant to 3 % (w/v) NaCl but could not grow at 5 % (w/v) NaCl. Strain K1^T was not obligately Na⁺-dependent: it grew in medium with K⁺ salts substituted in equimolar amounts for Na⁺ salts (three successive inoculations). Since strain K1^T was isolated from manure, it was important to study its requirement for NH₄⁺. The strain showed good growth at NH₄Cl concentrations of 0.1–0.5 % (w/v), with an optimum at 0.1 % (w/v) NH₄Cl, but not at 1 % (w/v) NH₄Cl. The temperature range for growth was 37–66 °C, with an optimum at 62 °C (Fig. 2b). Therefore, strain K1^T is a moderate thermophile.

In addition to D-glucose, strain K1^T was capable of fermenting sucrose, D-fructose, D-trehalose and starch (5 g l⁻¹). The major metabolic products were H₂ and acetate. No growth was observed with formate, acetate, propionate, pyruvate, lactate, butyrate, methanol, ethanol, glycerol, yeast extract, peptone, methylamine, casein, L-cysteine, L-valine, sorbitol, dulcitol, mannitol, inositol, L-sorbose, D-melezitose, L-rhamnose, DL-arabinose, D-galactose, D-melibiose, D-raffinose, D-ribose, D-lactose or DL-xylose.

Strain K1^T was able to reduce NaNO₃ to NaNO₂, but it could not use Na₂SO₄, Na₂SO₃, Na₂S₂O₃·5H₂O, S⁰ or benzenesulfonate as electron acceptors; no formation of H₂S was observed during growth in media containing sulfur compounds.

The bacterium studied was sensitive to bacitracin (at 100 µg l⁻¹), but resistant to penicillin, vancomycin, ampicillin and streptomycin (all at 250 µg ml⁻¹), and chloramphenicol (100 µg ml⁻¹).

Cellular fatty acids

The cellular fatty acid profile of strain K1^T (Table 1) differed (at a correlation level of $k > 0.5$) from that of 435 strains of various taxonomic groups, including 80 species of *Bacillus*, available in the database (obtained from one of the authors, G. Osipov, private collection). This implies that the studied bacterium cannot be assigned to any of the known species of the genus *Bacillus*.

Fig. 1. Electron micrographs of cells of strain K1^T. (a) Light micrograph (bar, 16.0 µm); (b) light micrograph of a branching form (bar, 3 µm); (c) ultrathin section (bar, 1.0 µm); (d) ultrathin section of the Gram-positive cell wall (*S*_{out}, outer S-layer, *S*_{in}, inner S-layer, PG, peptidoglycan layer; bar, 0.2 µm); (e) ultrathin section of a sporulating cell (*Ps* is a forespore; bar, 0.5 µm); (f) ultrathin section of a branching form (bar, 0.5 µm).

Fig. 2. Dependence of the growth of strain K1^T on temperature (a) and pH (b).

Table 1. Fatty acids of *Anoxybacillus pushchinensis* K1^T

The medium contained (g l⁻¹): KH₂PO₄, 0.2; MgCl₂·6H₂O, 0.1; KCl, 0.2; NH₄Cl, 1.0; Na₂CO₃, 2.76; NaHCO₃, 10.0; NaCl, 5.0; Na₂S·9H₂O, 0.5; yeast extract, 0.01 (Difco); and glucose, 5.0. The pH of the medium was of 9.5–9.7. The culture was grown at 55–62 °C.

Fatty acid	Percentage of total fatty acids in <i>Anoxybacillus pushchinensis</i> K1 ^T
C _{12:0}	6.9
C _{14:0}	7.3
iso-C _{15:0}	38.7
anteiso-C _{15:0}	2.0
C _{15:0}	0.9
iso-C _{16:0}	0.3
C _{16:1}	2.6
C _{16:0}	14.5
10Me-C _{16:0}	0.9
iso-C _{17:0}	0.8
anteiso-C _{17:0}	0.1
hydroxyiso-C _{15:0}	0.3
C _{17:0}	0.5
C _{18:2}	2.2
C _{18:1δ9}	4.3
C _{18:1δ1}	1.0
C _{18:0}	10.4
C _{20:0}	0.6

Genotypic characteristics

The G+C content of the DNA of strain K1^T was 42.2±0.2 mol%, a value similar to that of '*B. flavothermus*' DSM 2641 (41.6±0.2 mol%, as determined in this work). The level of DNA relatedness between these strains was 58.8%. The levels of DNA relatedness of strain K1^T with *Clostridium stercorarium* NCIMB 11754 (G+C content of 40.2 mol%), *Bacillus*

pasteurii VKM B 513 (G+C content of 38.5 mol%) and *Thermoanaerobacter thermohydrosulfuricus* DSM 567^T (G+C content of 37 mol%) were 21, 20 and 4%, respectively.

Phylogenetic analysis

A length of 1340 bp of 16S rDNA of strain K1^T was sequenced on both strands. The sequence was compared with the 16S rDNA sequences of some representatives of the *Bacillus* group by using the Phylogenetic Inference Package (version 3.5) (Felsenstein, 1989).

Phylogenetic analysis revealed a clusterization with '*B. flavothermus*' (98.89% sequence similarity). These bacteria differed by 7–16% from other species of the genus *Bacillus* and they can, therefore, be distinguished as a separate genus. Strain K1^T and '*B. flavothermus*' form an individual branch among the other bacillus rDNA groups. Table 2 shows the position of strain K1^T in the phylogenetic cluster of related bacteria.

DISCUSSION

Table 3 summarizes data of bacteria of the genera *Bacillus* and *Thermoanaerobacter*, which are most similar in their physiology to strain K1^T, despite some remarkable differences existing between them. Thus, strain K1^T differs from '*B. flavothermus*' in its morphology, cell size, motility (no peritrichous flagellation), and pH range and optimum. Unlike '*B. flavothermus*', strain K1^T cannot grow at 30 or 70 °C; its growth temperature ranges from 37 to 65 °C. In addition, strain K1^T is unable to utilize peptone and yeast extract as substrates, does not hydrolyse casein, cannot grow under aerobic conditions and is catalase-negative. Cells of *Thermoanaerobacter thermohydrosulfuricus* are motile, have a lower DNA G+C content than strain K1^T cells, exhibit a remarkably different range and optimum of temperatures and pH for growth, and are able to utilize yeast extract. Cells of *Bacillus pasteurii* (Sneath, 1986) are larger in size than cells of strain K1^T, exhibit different optimal and ultimate temperature and pH values, are tolerant to NaCl up to 10%, can hydrolyse gelatin and casein, and, finally, grow as facultatively anaerobic bacteria. It should be emphasized that strain K1^T can be distinguished from all other strains chosen for comparison by the obligate dependence of its growth on the CO₃²⁻ ion and a tendency toward the formation of branched cells. The 16S rDNA of strain K1^T was compared with GenBank database sequences using the FASTA program (Pearson & Lipman, 1988). The comparison revealed the highest degree of similarity of the strain with representatives of the genus *Bacillus sensu stricto* and related taxa. A strictly anaerobic species of the genus *Bacillus*, *Bacillus infernus*, has already been described (Boone *et al.*, 1995) but this species clearly belongs to a different cluster within this phyletic group. Comparisons of 16S rDNA sequences indicated the significant difference (> 80%) between strain K1^T and

Table 2. 16S rDNA difference values between strain K1^T and related taxa

Values for *Anoxybacillus pushchinensis* sp. nov. and *Anoxybacillus flavithermus* comb. nov. DSM 2641^T (Z26932) (formerly '*Bacillus flavothermus*') are shown in bold. Species are as follows (accession numbers are given in parentheses): 1, *Anoxybacillus pushchinensis* sp. nov.; 2, *Bacillus* sp. strain 13 DSM 2349 (Z26929); 3, *Bacillus pallidus* DSM 3670^T (Z26930); 4, '*Bacillus thermoalkalophilus*' DSM 6866 (Z26931); 5, *Alicyclobacillus acidocaldarius* DSM 446^T (X60742); 6, *Bacillus pasteurii* NCIB 8841^T (X60631); 7, *Anoxybacillus flavithermus* comb. nov. DSM 2641^T (Z26932) (formerly '*Bacillus flavothermus*'); 8, *Bacillus pseudofirmus* DSM 8715^T (X76439); 9, *Bacillus cereus* NCTC 11143 (55063); 10, *Bacillus alcalophilus* DSM 485^T (X76436); 11, *Bacillus pseudocaliphilus* DSM 8725^T (X76449); 12, *Bacillus gibsonii* DSM 8722^T (X76446); 13, *Bacillus clausii* DSM 8716^T (X76440); 14, *Bacillus megaterium* DSM 32^T (X60629); 15, *Bacillus horikoshii* DSM 8719^T (X76443); 16, *Aneurinibacillus aneurinolyticus* ATCC 12856^T (D78455); 17, *Bacillus brevis* NCIB 9372^T (X60612); and 18, *Thermoanaerobacter thermohydrosulfuricus* E100-69^T (L09161).

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
2	7.1																
3	7.2	0.1															
4	7.1	0.6	0.6														
5	18.4	17.5	17.5	17.9													
6	10.9	10.4	10.5	10.7	18.0												
7	1.1	6.1	6.3	6.6	17.1	9.5											
8	11.7	9.4	9.3	9.6	18.3	10.3	10.4										
9	10.6	9.4	9.4	9.4	20.0	10.7	9.7	7.2									
10	11.5	9.0	8.9	9.5	19.1	11.0	10.2	4.2	8.8								
11	11.3	8.6	8.7	9.1	19.8	11.4	10.4	4.8	8.4	2.1							
12	13.0	9.7	9.9	10.0	19.6	11.8	11.6	6.6	10.4	6.4	6.8						
13	12.2	8.0	8.2	8.5	19.1	11.3	10.9	6.3	9.5	5.8	6.1	6.0					
14	10.1	9.4	9.5	9.6	19.4	9.7	9.1	7.1	5.7	9.2	8.8	10.1	9.5				
15	13.0	9.7	9.9	10.0	19.6	11.8	11.6	6.6	10.3	6.4	6.8	0.0	6.0	10.1			
16	15.4	12.5	12.5	13.1	18.5	13.5	13.9	12.0	13.1	13.8	14.5	14.6	13.2	13.6	14.6		
17	14.3	11.6	11.6	12.1	18.7	12.0	13.0	11.9	12.6	11.7	11.7	12.1	11.7	12.0	12.1	10.3	
18	33.0	35.9	35.8	35.8	35.7	38.4	34.3	34.1	35.2	34.0	34.3	35.8	33.5	36.7	35.8	35.1	34.8

B. infernus. Due to this large difference, the latter species was not included in Table 2. '*B. flavothermus*' was the closest relative of strain K1^T, their 16S rDNA sequences exhibiting 98.8% similarity to each other. However, the 16S rDNA relatedness of strain K1^T and '*B. flavothermus*' is inconsistent with the phenotypic features which are clearly different for these two organisms. It is known that the analysis of 16S rDNA sequences may be insufficient to distinguish between species (Vandamme *et al.*, 1996). Therefore, DNA–DNA hybridization was performed, which showed 58.8% similarity between the two strains. This indicates that strain K1^T and '*B. flavothermus*' represent different species. Because 16S rDNA sequence similarity of these bacteria differed by 7–16% from those of other representatives of the genus *Bacillus* and its relatives, they can be distinguished as a separate taxon at the generic level (Tables 3 and 4). Based on the morphological and physiological features determined, as well as the data of genetic analyses, it is proposed that strain K1^T be placed in a new genus, *Anoxybacillus*, as a new species, *Anoxybacillus pushchinensis* gen. nov., sp. nov.

The aim of the present investigation was mainly the elucidation of the taxonomic structure of strain K1^T.

However, based on a comparison of physiological and chemotaxonomic data and genetic characterizations, including determination of G+C content of DNA, DNA–DNA hybridization and 16S rDNA analysis (our data; Heinen *et al.*, 1982; Claus & Berkeley, 1986; Ash *et al.*, 1991; Sharp *et al.*, 1992; Rainey *et al.*, 1994), this new genus should also include the species '*B. flavothermus*' (strain d.y = DSM 2641), the name having not been validly published. It is, therefore, proposed that '*B. flavothermus*' be placed also in the genus *Anoxybacillus* as *Anoxybacillus flavithermus* comb. nov.

Description of *Anoxybacillus* gen. nov.

Anoxybacillus (An.o.xy.ba.cil'lus. Gr. pref. *an* without; M.L. *oxy* shortened from *oxygenium*, oxygen; L. masc. n. *bacillus* small rod; N.L. masc. n. *Anoxybacillus* small rod living without oxygen).

Cells are rod-shaped and straight, 0.4–0.85 × 2.5–3.0 µm, often arranged in pairs or chains, with rounded ends. Gram-positive. Endospores are round and resistant to heating and freezing. Spores are located at the end of the cell. There is not more than one spore per cell. Obligately anaerobic or facultatively anaer-

Table 3. Comparison of *Anoxybacillus pushchinensis* K1^T with bacteria that have similar physiology and G + C content

Species are as follows: 1, *Anoxybacillus pushchinensis* strain K1^T (this work); 2, '*Bacillus flavothermus*' strain d.y. DSM 2641 (Heinen *et al.*, 1982); 3, *Thermoanaerobacter thermohydrosulfuricus* DSM 567^T (Klaushofer & Parkkinen, 1965); 4, *Bacillus pasteurii* VKM B-513 (Sneath, 1986).

Characteristic	1	2	3	4
Morphology				
Shape of cells	Rod	Rod	Rod	Rod
Motility	—	+	+	+
Spore formation	+	+	+	+
Size (µm)	0.5–0.6 × 3.0–5.0	0.85 × 2.3–7.1	0.4–0.6 × 1.8–13.0	0.5–1.2 × 1.3–4.0
DNA G + C (mol %)	42.2	42.3/61.0*	37	38.5–42.0
Temperature (°C):				
Range	37–66	30–72	42–78	15–40
Optimum	62	60–65	67–69	30
pH:				
Range	8.0–10.5	5.5–9.0	5.5–9.2	6.5–9.5
Optimum	9.5–9.7	7.0	6.9–7.5	9.0
Tolerance to NaCl (%)	3.0	2.5	ND	10
Substrate:				
Glucose	+	—	+	+
Sucrose	+	—	+	+
Starch	+	—	+	ND
Peptone	—	+	—	+
Yeast extract	—	+	+	ND
Lactate	—	—	—	ND
Relation to O ₂	Anaerobe	Facultative aerobe	Anaerobe	Facultative aerobe
Catalase activity	—	+	—	ND
NO ₃ ⁻ reduction	+	+	+ / —	+
Hydrolysis of gelatin	—	—	—	+
Hydrolysis of casein	—	+	+ / —	+

* G + C values of 42.3 and 61.0 mol % from H. Hippe (DSMZ) and Heinen *et al.* (1982), respectively.

Table 4. Comparison of *Anoxybacillus* gen. nov. with genera that have similar endospore formation

Genera are as follows: 1, *Anoxybacillus*; 2, *Amphibacillus*; 3, *Bacillus*; 4, *Clostridium*; 5, *Desulfotomaculum*; and 6, *Sporolactobacillus*. All genera are rod-shaped and produce endospores.

Characteristic	1	2	3	4	5	6
Motility	— / +	+	+	+	+	+
Gram reaction	+	+	+	+ / — (rare)	+	+
Relation to O ₂	Anaerobe/ facultative aerobe	Facultative aerobe	Anaerobe/ facultative aerobe	Anaerobe (aerotolerant)	Obligate anaerobe	Anaerobe/ facultative aerobe
SO ₄ ²⁻ reduction	—	—	—	—	+	—
Catalase activity	+ / —	—	+	—	—	—
Oxidase activity	+ / —	—	+ / —	—	—	—
NO ₃ ⁻ reduction to NO ₂ ⁻	+	—	+ / —	+ / —	—	—
NaCl requirement (3–12 %)	—	—	+ / —	—	—	—
Lactate as sole product of carbohydrate fermentation	—	—	+ / —	—	—	+

obic; catalase variable. Alkaliphilic or alkalitolerant, thermophilic. Chemo-organotrophic, with a fermentative metabolism. DNA G+C content is around 42 mol%. Includes two species: *Anoxybacillus pushchinensis* and *Anoxybacillus flavithermus*. The type species of the genus is *Anoxybacillus pushchinensis*.

Description of *Anoxybacillus pushchinensis* sp. nov.

Anoxybacillus pushchinensis (push.chi.nen'sis. N.L. masc. adj. *pushchinensis* pertaining to Pushchino, a research centre near Moscow, Russia, where the organism was isolated).

Straight rods, 0.4–0.5 × 2.5–3.0 µm in size, single, in pairs, sometimes in chains, Gram-positive, non-motile. Y-shaped cells occur. Forms round endospores. Anaerobic, chemoheterotrophic, alkaliphilic, moderately thermophilic bacterium. Grows at 37–65 °C, with an optimum at 62 °C. Obligate alkaliphile that cannot grow at pH 7.0; grows in a pH range of 8.0–10.5 with an optimum at 9.5–9.7. CO₃²⁻ is obligately required. Optimal growth at 1% NaCl, tolerant to 3% NaCl. Growth substrates are D-glucose, sucrose, D-fructose, D-trehalose and starch. The major fermentation products are hydrogen and acetic acid. Nitrate is reduced to nitrite. Sulfate, sulfite, thiosulfate and sulfur are not reduced. Yeast extract stimulates growth. Vitamins are required. Catalase-negative. Gelatin and casein are not hydrolysed. DNA G+C content is 42.2 ± 0.2 mol%. Source: cow and pig manure with neutral pH. Type strain: K1^T. This strain has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (as DSM 12423^T), at the American Type Culture Collection (as ATCC 700785^T) and at the All-Russia Collection of Microorganisms (as VKM B-2193^T).

Description of *Anoxybacillus flavithermus* comb. nov.

Anoxybacillus flavithermus (fla.vi.ther'mus. L. adj. *flavus* yellow; Gr. adj. *thermos* warm; N.L. adj. *flavithermus* to indicate a yellow thermophilic organism).

Data are from Heinen *et al.* (1982), Claus & Berkeley (1986), Sharp *et al.* (1992), Rainey *et al.* (1994) and this study. Rods, 0.85 × 2.3–7.1 µm. Motile. Gram-positive. Spores terminal. Colonies round, smooth, yellow. Facultatively anaerobic. Catalase-positive. Oxidase-positive. Starch but not gelatin hydrolysed. Grows in peptone-yeast extract media. Glucose, mannose, maltose, sucrose, arabinose, rhamnose and sorbitol utilized. Positive for acetoin, arginine dihydrolase, lysine decarboxylase, tryptophan deaminase and β-galactosidase. Nitrate reduced to nitrite. Urease, ornithine decarboxylase, indole and H₂S not produced. Growth in 2.5% NaCl broth, but not in 3% NaCl. Optimum pH for growth of 6–9. No growth at pH 5.0. Temperature range for growth 30–72 °C; optimum growth at 60 °C (aerobic) and 65 °C (anaerobic). Source: hot spring, New Zealand. DNA G+C content

of 41.6 mol% (by HPLC method). Type strain: d.y.^T (= DSM 2641^T).

ACKNOWLEDGEMENTS

We are grateful to Dr A. V. Lebedinskyi and Dr A. L. Mulukin for editing the English version of this article. This work was in part supported by the RFFI grant 99-04-49144.

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