Bacillus butanolivorans sp. nov., a species with industrial application for the remediation of n-butanol

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Four bacterial strains, designated K9^T, K105, K1012A and K101, were isolated from soil in Lithuania. All these strains could use n-butanol as a sole carbon source. The strains grew in a medium containing 12–120 mM n-butanol. The strains were strictly aerobic, Gram-positive endospore-formers. The best growth was achieved at 25 °C and pH 7.0 in medium containing 1 % (w/v) NaCl. The strains showed identical profiles of 16S–23S rRNA internal transcribed spacer PCR and nearly identical 16S rRNA gene PCR-RFLP electrophoretic patterns and physiological characteristics, demonstrating their relationship at the species level. The cellular fatty acid profile of K9^T consisted of significant amounts of the C₁₅ branched-chain fatty acids iso-C_{15:0} (16.78 %) and anteiso-C_{15:0} (45.80 %). The diagnostic cell-wall diamino acid was meso-diaminopimelic acid. The 16S rRNA gene sequence of K9^T showed the highest similarity to the sequences of *Bacillus simplex* DSM 1321^T and *Bacillus muralis* LMG 20238^T (98.3 and 97.7 %, respectively). The DNA G + C content was 37.4 mol%. Studies of DNA–DNA relatedness, morphological, physiological and chemotaxonomic analyses and phylogenetic data based on 16S rRNA gene sequencing allowed strains K9^T, K105, K1012A and K101 to be described as members of a novel species of the genus *Bacillus*, for which the name *Bacillus butanolivorans* sp. nov. is proposed. The type strain is K9^T (=DSM 18926^T =LMG 23974^T).

n-Butanol (1-butanol, butan-1-ol) is released into the environment from natural and anthropogenic sources. It is defined as a volatile organic compound. As such, n-butanol can contribute to the formation of photochemical smog when it reacts with other volatile compounds in the atmosphere. n-Butanol present in the environment undergoes complex chemical reactions and may cause numerous indirect effects, particularly the formation of photochemical oxidants and their main constituent, ozone. High concentrations of ozone in the air can impair human health. Because of its potential health effects and influence on photochemical smog, removal of n-butanol from industrial waste streams is highly desirable (Veeranagouda *et al.*, 2006).

In a screening campaign aimed at isolating strains with the ability to tolerate high concentrations of n-butanol and to use it as a sole carbon source, strains K9^T, K105, K1012A and K101 were isolated from soil sampled near the city of Klaipėda in Lithuania. The sampling depth was 0.02 m. All the strains originated from two different samples. The strains were isolated on minimal medium (Arp, 1999) supplemented with a solution of microelements (Lageveen *et al.*, 1988). n-Butanol (12 mM) was used as the sole carbon source. The isolated strains could tolerate 12–120 mM n-butanol; 240 mM was lethal to these strains. Strains K9^T, K105, K1012A and K101 were further cultivated and maintained on nutrient agar.

For morphological characterization, the strains were cultivated at 25 °C on nutrient agar for 24 h. Both the morphology of cells and the characteristics of colonies were investigated. Cell morphology was examined under an Olympus AX70 microscope (magnification ×1000) and a JEM-100S electron microscope (magnification ×3000–10 000). For bright-field microscopy, cells were stained using Gram staining. For electron microscopy, cells were

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain K9^T is EF206294.

ARDRA and RISA profiles for the novel strains are available as supplementary material with the online version of this paper.

prepared as described by Mignot *et al.* (2001). The presence and morphology of endospores were checked in cells grown on nutrient agar for 2 weeks. Results of morphological characterization were identical for all the strains investigated and are given in the species description.

Genotyping experiments were repeated twice using different DNA extractions for amplification and different amplification products for restriction analysis. Results of these completely independent experiments were identical.

Ribosomal intergenic spacer analysis (RISA) was performed with primers S-D-Bact-1494-a-S-20 and L-D-Bact-0035-a-A-15 (Daffonchio *et al.*, 1998) as described by Kuisiene *et al.* (2002). RISA profiles were analysed by electrophoresis through 5% polyacrylamide gel. Strains K9^T, K105, K1012A and K101 showed identical RISA profiles (see Supplementary Fig. S1, available in IJSEM Online). It can be concluded that strains K9^T, K105, K1012A and K101 belong to a single species.

Amplified rDNA restriction analysis (ARDRA) was performed in order to investigate further the genomic diversity of strains K9^T, K105, K1012A and K101. Restriction analysis was performed with *AluI*, *HaeII*, *HaeIII* and *TaqI* as described by Kuisiene *et al.* (2002). Strain K1012A differed in a single band in the *TaqI* profile. The patterns of *AluI*, *HaeII* and *HaeIII* were identical for all the strains, confirming the close genetic relationship between these strains. Electrophoretic patterns of ARDRA are shown in Supplementary Figs S1 and S2.

All physiological assays were repeated three times. The temperature range for growth was determined in nutrient broth buffered with 50 mM Tris/HCl (pH 7.0) by measuring the OD₆₀₀. In order to study the influence of pH on bacterial growth, the 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, 8.8). Bacterial growth in buffered medium was monitored by measuring the OD₆₀₀ using a Beckman DU-650 spectrophotometer. Most physiological tests were carried out using methods described by Claus & Berkeley (1986). Cultures were incubated at 25 °C for 24 h. The physiological characteristics were nearly identical for all the strains tested, confirming that strains K9^T, K105, K1012A and K101 belong to a single species. The strains differed in the following characteristics: hydrolysis of casein (K9^T and K105 hydrolysed casein, while K1012A and K101 did not) and assimilation of *myo*-inositol, L-rhamnose and raffinose. Strain K9^T did not assimilate and strain K1012A grew weakly on these carbon sources. Results of the physiological characterization are given in the species description and in Table 1.

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 the agarose gel using a DNA extraction kit (Fermentas), and the purified PCR product was cloned into *Escherichia coli*

DH5α using the InsT/AcloneTM PCR product cloning kit (Fermentas). Recombinant clones were detected through blue/white screening (Sambrook *et al.*, 1989). Recombinant plasmid DNA was extracted as described by Birnboim & Doly (1979).

Cloned 1.5 kb DNA fragments amplified by PCR were sequenced by automated DNA sequencing. 16S rRNA gene sequences were edited and sequence similarity was determined using the SEQBUILDER and MEGALIGN components of LASERGENE 6 (DNASTAR). The 16S rRNA gene sequences of the tested strains were aligned using the MEGA 3.1 program (Kumar *et al.*, 2004); 1471 nucleotides were used for the alignment. A phylogenetic tree was constructed using the MEGA 3.1 program by the neighbour-joining method (Saitou & Nei, 1987). The pairwise-deletion option was used. 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 sequence of *Brevibacillus brevis* NBRC 15304^T as an outgroup. The phylogenetic tree (Fig. 1) shows the phylogenetic position of strain K9^T among closely related species of the genus *Bacillus*.

The 16S rRNA gene sequence of strain K9^T was most similar to those of *Bacillus simplex* DSM 1321^T and *Bacillus muralis* LMG 20238^T (98.3 and 97.7%, respectively). Sequence similarity with *Bacillus asahii* MA001^T and *Bacillus psychrosaccharolyticus* ATCC 23296^T was 96.1 and 95.4%, respectively, indicating no relatedness between K9^T and any of them at the species level. Sequence similarity with other representatives of the genus *Bacillus* was below 95.0%.

On the basis of 16S rRNA gene sequence similarity, *B. simplex* DSM 1321^T and *B. muralis* DSM 16288^T were chosen for DNA–DNA hybridization experiments. For determination of G+C content and for DNA–DNA hybridization, DNA was isolated by breaking cells in a French press, followed by purification by chromatography on hydroxyapatite according to the procedure of Cashion *et al.* (1977). The G+C content of strain K9^T was determined by HPLC (Mesbah *et al.*, 1989) as 37.4 mol%.

DNA–DNA hybridization was performed under optimal conditions (2 × SSC at 63 °C) using a Cary 100Bio spectrophotometer. DNA–DNA relatedness of strain K9^T was 29.35% with *B. simplex* DSM 1321^T and 28.75% with *B. muralis* DSM 16288^T. The results of DNA–DNA hybridization as well as those of phylogenetic analysis showed that strain K9^T belongs to the genus *Bacillus*, but represents a novel species within this genus.

Chemotaxonomic characteristics of strain K9^T were also in agreement with the properties of the genus *Bacillus*. The diagnostic cell-wall diamino acid was *meso*-diaminopimelic acid, determined as described by Schleifer (1985). Cells for cellular fatty acid analysis were harvested from 24 h cultures grown at 28 °C on tryptone soya agar. Fatty acids

Table 1. Differentiating characteristics of strain K9^T and the phylogenetically most closely related species of the genus *Bacillus*

Reference species: 1, *B. simplex* (data from Heyrman *et al.*, 2005 unless indicated); 2, *B. muralis* (Heyrman *et al.*, 2005); 3, *B. psychrosaccharolyticus* (Priest *et al.*, 1988); 4, *B. asahii* (Yumoto *et al.*, 2004). +, Positive or >85 % of strains positive; v, variable (16–84 % positive); –, negative or 0–15 % of strains positive; w, weakly positive; v/w, variable and, when positive, weak; bv., biovar; NG, no growth; ND, no data available. All species were motile and negative for indole production.

Characteristic	Strain K9 ^T	1	2	3	4
Pink pigmentation	–	–	+	–	–
Sporangia swollen	–	–	w	+	–
Growth in/at/on:					
5 °C	+	–	–	+	–
45 °C	+	–	–	–	ND
MacConkey agar	–	+	ND	+	–
2 % (w/v) NaCl	+	+	+	+	–
5 % (w/v) NaCl	+	v	+	v	–
7 % (w/v) NaCl	–	–	w	ND	–
Catalase	+	+	+	ND	+
Hydrolysis of:					
Casein	–	v	v	+	+
Starch	–	+	+	+	w
Gelatin	–	v	–	+	–
Pigment from casein	–	Brown	Salmon pink	ND	ND
Nitrate reduction	+	+	+	+	w
Decomposition of tyrosine	+	ND	ND	–	ND
Voges–Proskauer test	–	–	–	v	–
Production of acid from:					
Cellobiose	–	– (bv. 1); v/w (bv. 2)	+	v	–
Dulcitol	NG	–	–	–	ND
Fructose	–	+	+	+	–
Galactose	NG	–	+	–	–
Glucose	–	+	+	+	–
Lactose	NG	–	+	–	–
Maltose	–	– (bv. 1); v/w (bv. 2)	+	+	–
Mannitol	–	v/w (bv. 1); w (bv. 2)	+	v	–
Mannose	–	–	+	+	–
Ribose	–	v/w (bv. 1); w (bv. 2)	+	ND	ND
Sorbitol	–	v/w (bv. 1); w (bv. 2)	–	v	–
Sucrose	–	+	v	v	–
D-Xylose	–	– (bv. 1); w (bv. 2)	–	+	–
Fatty acids (% of total)					
iso-C _{15:0}	16.78	15.55 ± 2.95	22.51 ± 4.47	ND	39.0
anteiso-C _{15:0}	45.80	59.03 ± 5.88	42.69 ± 5.72	ND	27.8
C _{16:1} ω7c alcohol	5.79	2.97 ± 0.74	4.11 ± 1.67	ND	ND
iso-C _{16:0}	4.48	2.26 ± 1.42	1.61 ± 1.00	ND	ND
anteiso-C _{17:0}	2.72	1.82 ± 0.80	<1.0	ND	ND
G + C content (mol%)	37.4	39.5–41.8	41.2	43–44	39.4

*Data from Yumoto *et al.* (2004).

were extracted and analysed following the instructions of the Microbial Identification System operating manual (MIDI, 1999). In general, the fatty acid profile of strain K9^T was similar to those of *B. simplex*, *B. muralis* and *B. asahii*. iso-C_{15:0} and anteiso-C_{15:0} represent the main fatty acids of these species as well as numerous species within the bacilli. Strain K9^T contained a smaller amount of iso-C_{15:0} and a larger amount of anteiso-C_{15:0} than *B. muralis* and

B. asahii. C₁₅ branched-chain fatty acids made up 62.58 % of the total fatty acid content in strain K9^T. This value was the lowest when comparing K9^T with *B. simplex*, *B. muralis* and *B. asahii* (Table 1). Larger amounts of C_{16:1}ω7c alcohol, iso-C_{16:0} and anteiso-C_{17:0} fatty acids differentiated between K9^T and the species listed above. The relative fatty acid concentrations are listed below in the species description.

Fig. 1. Phylogenetic position of strain K9^T among other *Bacillus* species on the basis of 16S rRNA gene sequences, determined by using the neighbour-joining method. Numbers at nodes represent percentage bootstrap values obtained from 1000 samplings. Only values greater than 70% are shown. *Brevibacillus brevis* NBRC 15304^T was defined as the outgroup of the tree. Bar, 0.01 nucleotide substitutions per site.

On the basis of the above results, we conclude that strains K9^T, K105, K1012A and K101 represent a novel species of the genus *Bacillus*. We describe K9^T as the type strain of this novel species, for which we propose the name *Bacillus butanolivorans* sp. nov.

Description of *Bacillus butanolivorans* sp. nov.

Bacillus butanolivorans (bu.ta.no.li.vo'rans. N.L. neut. n. *butanolium* butanol; L. part. adj. *vorans* devouring, consuming; N.L. part. adj. *butanolivorans* butanol-consuming).

Gram-positive cells are rod-shaped and motile and occur in long chains and sometimes singly, varying in length from 2.5 to 5.1 µm and in diameter from 0.8 to 1.3 µm. Oval central endospores are produced within unswollen sporangia. Colonies, grown for 2 days on nutrient agar at 25 °C, are 2–4 mm in diameter, round, tawny, slightly raised and opaque. Strictly aerobic. The growth temperature range is 5–45 °C, with an optimum at 25 °C. Growth occurs at pH 6.0–8.8, with an optimum at pH 7.0. Growth is observed in the presence of 0.5–5% (w/v) NaCl, with an optimum at 1% (w/v) NaCl. Phenylalanine is not deaminated and growth in Sabouraud dextrose broth is negative. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase reactions are negative. L-Arabinose is not assimilated; utilization of *myo*-inositol, raffinose and L-rhamnose is variable. The diamino acid in the cell wall is *meso*-diaminopimelic acid. The fatty acid profile consists of iso-C_{13:0} (0.29% in the type strain), iso-C_{14:0} (8.77%), C_{14:0} (1.76%), iso-C_{15:0} (16.78%), anteiso-C_{15:0} (45.80%), C_{15:0} (1.33%), C_{16:1ω7c} alcohol (5.79%), iso-C_{16:0} (4.48%), C_{16:1ω11c} (6.14%), C_{16:0} (2.97%), iso-C_{17:1ω10c} (0.97%), iso-C_{17:1} I and/or anteiso-C_{17:1} B (0.94%), iso-C_{17:0} (1.27%) and anteiso-C_{17:0} (2.72%). Differentiating characteristics are indicated in Table 1. The DNA G+C content of the type strain is 37.4 mol%.

Known strains have been isolated from soil. The type strain is K9^T (=DSM 18926^T =LMG 23974^T). In the characters

for which a variable result was obtained, the type strain does not hydrolyse casein and does not assimilate *myo*-inositol, L-rhamnose or raffinose.

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