Proposal of *Lysinibacillus boronitolerans* gen. nov. sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and *Bacillus sphaericus* to *Lysinibacillus sphaericus* comb. nov.

Iftikhar Ahmed,^{1,2} Akira Yokota,³ Atsushi Yamazoe⁴ and Toru Fujiwara^{1,5}

Correspondence

Iftikhar Ahmed
iftikharnarc@hotmail.com

Toru Fujiwara
atorufu@mail.ecc.u-tokyo.ac.jp

¹Biotechnology Research Center, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

²National Agricultural Research Centre, Park Road, Islamabad – 45500, Pakistan

³Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

⁴Research Center for Water Environmental Technology, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8657, Japan

⁵SORST, JST, Chiyoda-ku, Tokyo, Japan

Three strains of a spore-forming, Gram-positive, motile, rod-shaped and boron-tolerant bacterium were isolated from soil. The strains, designated 10a^T, 11c and 12B, can tolerate 5 % (w/v) NaCl and up to 150 mM boron, but optimal growth was observed without addition of boron or NaCl in Luria–Bertani agar medium. The optimum temperature for growth was 37 °C (range 16–45 °C) and the optimum pH was 7.0–8.0 (range pH 5.5–9.5). A comparative analysis of the 16S rRNA gene sequence demonstrated that the isolated strains were closely related to *Bacillus fusiformis* DSM 2898^T (97.2 % similarity) and *Bacillus sphaericus* DSM 28^T (96.9 %). DNA–DNA relatedness was greater than 97 % among the isolated strains and 61.1 % with *B. fusiformis* DSM 2898^T and 43.2 % with *B. sphaericus* IAM 13420^T. The phylogenetic and phenotypic analyses and DNA–DNA relatedness indicated that the three strains belong to the same species, that was characterized by a DNA G + C content of 36.5–37.9 mol%, MK-7 as the predominant menaquinone system and iso-C_{15:0} (32 % of the total) as a major cellular fatty acid. In contrast to the type species of the genus *Bacillus*, the strains contained peptidoglycan with lysine, aspartic acid, alanine and glutamic acid. Based on the distinctive peptidoglycan composition, phylogenetic analyses and physiology, the strains are assigned to a novel species within a new genus, for which the name *Lysinibacillus boronitolerans* gen. nov., sp. nov. is proposed. The type strain of *Lysinibacillus boronitolerans* is strain 10a^T (= DSM 17140^T = IAM 15262^T = ATCC BAA-1146^T). It is also proposed that *Bacillus fusiformis* and *Bacillus sphaericus* be transferred to this genus as *Lysinibacillus fusiformis* comb. nov. and *Lysinibacillus sphaericus* comb. nov., respectively.

Boron is an essential micronutrient for plants (Warington, 1923) and possibly also for animals (Rowe *et al.*, 1998; Rowe & Eckhert, 1999). In bacteria, Chen *et al.* (2002) presented evidence for the biological role of boron in quorum sensing. On the other hand, boron is toxic to living cells when present above a certain threshold. Because of its toxic effects at high

concentrations for micro-organisms, boron has been used as a food preservative (Nielsen, 2004), and it has also been used as an insecticide against cockroaches (Cochran, 1995). However, only recently, *Bacillus boroniphilus*, *Chimaericella boritolerans* and *Gracilibacillus boracitolerans* have been reported to tolerate more than 450, 300 and 450 mM boron, respectively, and/or require boron for growth (Ahmed *et al.*, 2007a, b, c).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 10a^T, 11c and 12B are respectively AB199591–AB199593.

Results of growth experiments in the presence of boron, photomicrographs of cells of strain 10a^T and results of separation of polar lipids of strain 10a^T and related type strains are available as supplementary material in IJSEM Online.

Over the last decade, the genus *Bacillus* has been dissected taxonomically into several new genera based on comprehensive analyses of 16S rRNA gene sequences and other chemotaxonomic data (Wisotzkey *et al.*, 1992; Ash *et al.*, 1993; Shida *et al.*, 1996; Heyndrickx *et al.*, 1998; Wainø *et al.*,

1999; Yoon *et al.*, 2001, 2002). rRNA group 2 of the genus *Bacillus* (Ash *et al.*, 1991) containing L-lysine or D-ornithine in the peptide subunit also includes non-*Bacillus*-type organisms such as members of *Caryophanon*, *Kurthia*, *Sporosarcina*, *Planococcus* and *Filibacter* (Clausen *et al.*, 1985; Keddie & Shaw, 1986; Stackebrandt *et al.*, 1987; Pechman *et al.*, 1976; Farrow *et al.*, 1994). There is a need to re-evaluate the taxonomic status of this group. Although it has been discussed previously (Farrow *et al.*, 1994; Rheims *et al.*, 1999), no proposal has yet been put forward to transfer these organisms into one or more new or existing genera. In this paper, we characterize three round-spore-forming strains belonging to a new genus in *Bacillus* RNA group 2. This genus is distinguished from other members of this group by the presence of lysine and aspartate in the peptidoglycan of the cell wall.

The three strains were isolated from soil sampled randomly from the experimental field of the University of Tokyo (Yayoi campus), Japan. We used previously described isolation and enrichment procedures (Ahmed *et al.*, 2007a). The purified isolates (strains 10a^T, 11c and 12B) were maintained on agar medium, and also stored as glycerol (35 %, w/v) stocks at -80 °C, and subjected to characterization.

To demonstrate boron tolerance, strain 10a^T and *Escherichia coli* DH10B (control) were grown up to upper mid-exponential phase (OD₆₀₀ 1.2) at 37 °C, with vigorous shaking. The cultures were serially diluted and 7 µl aliquots were spotted onto nutrient agar medium (NA; Difco) (pH 7.0) containing different levels of boron. The plates were incubated at 37 °C for 2 days before being photographed. Strain 10a^T manifested tolerance of boron up to 100 mM on NA, whereas weak growth was observed at 150 mM boron (Supplementary Fig. S1 in IJSEM Online). The strain was also cultured in 150 ml Luria-Bertani (LB) medium (pH 7.0) with different levels of boron ranging from 0 (control) to 300 mM, while being shaken vigorously at 30 °C. After every 60 min, samples were taken aseptically to measure OD₆₀₀ using a spectrophotometer (Hitachi U-1800). The data for OD₆₀₀ against time plotted at different boron concentrations demonstrated that strain 10a^T could grow with 0–100 mM boron in liquid culture (LB medium) and that growth was equivalent to that without addition of boron (Supplementary Fig. S2), indicating that boron was not required for growth. However, there was no growth at 200 mM boron and weak growth was observed at 150 mM boron.

Sporangia and the sizes of cells grown on NA with MgSO₄·7H₂O (1.01 mM), KCl (13.4 mM), FeSO₄ (0.001 mM), Ca(NO₃)₂ (1.0 mM) and MnCl₂ (0.01 mM) at pH 7.0 for 6 days at 30 °C were examined under phase-contrast microscopy. Cells of the strains produced oval or spherical endospores in a terminal position in a swollen sporangium (Supplementary Fig. S3). Cells of the strains were Gram-positive as determined according to Hucker's modified method (Cowan, 1974). Colonial morphology was observed on isolated colonies grown on nutrient agar

(pH 7.0, Difco) for 2 days at 30 °C. The pH range for growth was determined in tryptic soya broth (TSB; Difco) with a pH range of 4.0 to 10.0 at 30 °C by monitoring OD₆₀₀ using a mini-photometer (model 518R; TAITEC) and the temperature range for growth was determined on tryptic soya agar (TSA; Difco) (pH 7.0) by incubating at different temperatures from 4 to 50 °C. The isolated strains grew at pH 5.5–9.5 with optimum growth at pH 7.5 and no growth at pH 5.0. We observed growth of the strains at 16–45 °C with optimum growth at 37 °C; there was no growth at ≥50 °C and only slight growth after several days at 16 °C. These findings distinguished the strains from the closely related species *Bacillus fusiformis* and *Bacillus sphaericus*, which can only grow up to 40 °C. Growth at various NaCl concentrations was investigated on TSA (pH 7.0) at 30 °C. The novel strains tolerated 0–5 % (w/v) NaCl in the agar medium. This differed from *B. fusiformis*, which can tolerate up to 7 % (w/v) NaCl (Priest *et al.*, 1988). We also observed growth of the novel strains on marine agar 2216 (Difco), TSA and NA with and without addition of boron or NaCl.

Physiological and biochemical characteristics were determined using API 20E and API 50CH galleries (bioMérieux). The strains were positive for oxidase and catalase activities as assessed by previously described procedures (Ahmed *et al.*, 2007a). Motility was also confirmed with M medium (bioMérieux) in addition to microscopy. Since mainly negative reactions were obtained with API 50CH and API 20E for utilization of various carbon sources, we analysed an extended array of metabolic features of the strains using the Biolog GP2 and GN2 characterization system. Resistance to antibiotics was assessed with an ATB-VET strip (bioMérieux) and enzyme activities were determined with an API ZYM strip (bioMérieux). All commercial kits were used according to the manufacturers' protocols. The isolated strains exhibited many features that were similar to those of *B. fusiformis* and *B. sphaericus*; however, they differed from these species in certain physiological and biochemical characteristics (Table 1). All three novel strains gave identical results in these tests.

Nearly complete 16S rRNA gene sequences of the strains were amplified by the PCR as described by Katsivela *et al.* (1999). The purified PCR product was sequenced as described previously (Ahmed *et al.*, 2007a). The DNASIS Pro (Hitachi Software Engineering) software package was used to obtain the consensus sequence. Sequences of closely related type strains used for constructing the phylogenetic tree were selected and retrieved from the DDBJ/EMBL databases by BLAST searches for bacteria. Alignment was performed with CLUSTAL X (version 1.8w; Thompson *et al.*, 1997) and evolutionary distances and *K*_{nuc} values (Kimura, 1980) were generated. BioEdit software (Hall, 1999) was used to remove gaps and ambiguous bases in the alignments. A phylogenetic tree was constructed from 1294 unambiguously aligned nucleotides using the neighbour-joining method (Saitou & Nei, 1987) contained in the PHYLIP software package (Felsenstein, 2005) and plotted with NJPlot

software. The stability of the relationship was assessed by bootstrap analysis (Felsenstein, 2005) by performing 1000 resamplings for the tree topology of the neighbour-joining data.

An almost-complete 16S rRNA gene sequence (1484 nucleotides) of strain 10a^T was compared with sequences of closely related type strains retrieved from the public DDBJ database. Based on 16S rRNA gene sequence data, the similarity of the novel strain 10a^T was 97.2 % to *B. fusiformis* DSM 2898^T (GenBank accession no. AJ310083), 96.9 % to *B. sphaericus* DSM 28^T (AJ310084), 96.1 % to *Bacillus odyseeyi* 34hs-1^T, 95.0 % to *Bacillus massiliensis* 4400831^T and 94.5 % to *Bacillus silvestris* HR3-23^T; the similarity was 99.3 % with strain 11c and 99.2 % with strain 12B after alignment, whereas strains 11c and 12B had 99.8 % similarity. The 16S rRNA gene sequence of strain 11c was omitted during construction of the phylogenetic tree because the sequence was incomplete. The novel strains occupied a separate lineage in the phylogenetic tree with high bootstrap support (Fig. 1). Strain 10a^T is closely related to *B. fusiformis* and *B. sphaericus* in many other characteristics, although the data presented here also exhibited differences from these and other type strains of the genus *Bacillus* (Table 1) and among closely related genera (Table 2). On the basis of morphological, physiological, phylogenetic, chemotaxonomic and genomic characteristics which we determined, strains 10a^T, 11c and 12B are considered to be members of the same species.

For DNA–DNA hybridization, DNA from the isolated strains and closely related type strains was isolated using a previously described procedure (Ahmed *et al.*, 2007a). DNA–DNA hybridization was performed at 42 °C with photobiotin-labelled DNA and microplates as described by Ezaki *et al.* (1989), using an HTS7000 Bio Assay Reader (Applied Biosystems) for fluorescence measurements. The DNA–DNA hybridization values among strains 10a^T, 11c and 12B were more than 97.8 %, confirming that these strains belong to the same species. However, DNA–DNA relatedness of strain 10a^T was 61.1 % with *B. fusiformis* DSM 2898^T, 43.2 % with *B. sphaericus* IAM 13420^T and 26.4 % with *B. silvestris* DSM 12223^T. These values are less than the 70 % threshold needed to assign the strains to a novel species (Stackebrandt & Goebel, 1994). The DNA G + C content of the novel strains was 36.5–37.9 mol% as determined by HPLC using a procedure described previously (Ahmed *et al.*, 2007a).

Respiratory quinones were analysed as described by Xie & Yokota (2003) and MK-7 (87 %) was determined as the predominant quinone system in the novel strains, although MK-6 (13 %) was also detected as a minor component. The purified cell wall was analysed for amino acids using two-dimensional TLC and then HPLC (Shimadzu) as described previously (Schleifer & Kandler, 1972; Groth *et al.*, 1996). Strain 10a^T contained peptidoglycan with alanine, glutamic acid, lysine and aspartic acid in a molar ratio of 1.83:1.0:0.69:0.63 as the diagnostic amino acids, in

contrast to the type species of the genus *Bacillus*, *Bacillus subtilis*, which was diagnosed with *meso*-diaminopimelic acid in the cell-wall peptidoglycan (Schleifer & Kandler, 1972). During this study, *B. fusiformis* DSM 2898^T produced similar results in cell-wall peptidoglycan analysis, with lysine, alanine, glutamic acid and aspartic acid in a similar molar ratio to strain 10a^T. This represents peptidoglycan type A4α (Lys–Asp) as described by Schleifer & Kandler (1972). The close relative *B. sphaericus* was reported to contain L-Lys–D-Asp in the peptidoglycan as the diagnostic amino acids by Stackebrandt *et al.* (1987). So far, peptidoglycan consisting of Lys–Asp has not been reported for any other endospore-forming species of *Bacillus* group 2 (Ash *et al.*, 1991; Stackebrandt *et al.*, 1987; Nakamura *et al.*, 2002; Ranftl & Kandler, 1970; Rheims *et al.*, 1999; Claus & Fritze, 1989), but the closely related species of *Kurthia* (Fig. 1) share this characteristic (Shaw & Keddie, 1983).

For whole-cell fatty acid analysis, the cells were grown on TSA for 24 h at 28 °C and the cellular fatty acid profile was determined using the GC-based Microbial Identification system (MIDI) according to the manufacturer's instructions. The cellular fatty acid profile for the novel strains consisted predominantly of iso- and anteiso-branched fatty acids (Table 3), which is similar to other members of the *B. sphaericus*-like group. A MIDI database search of profiles also supported the novel species status of the strains, as there was no match to any known species. Polar lipids were extracted and purified from 100 mg dried cells of strain 10a^T, *B. fusiformis* DSM 2898^T and *B. sphaericus* IAM 13420^T by the procedure of Minnikin *et al.* (1984) and examined by two-dimensional TLC, using Kieselgel 60 F₂₅₄ plates (Merck), as described by Kudo (2001). Strain 10a^T shared a similar polar lipid profile with *B. fusiformis* DSM 2898^T and *B. sphaericus* IAM 13420^T, which consisted predominantly of diphosphatidylglycerol, phosphatidylglycerol and ninhydrin-positive phosphoglycolipid (Supplementary Fig. S4). The chemotaxonomic data showed some significant differences when compared with members of closely related genera, particularly the type species of the genus *Bacillus* in terms of polar lipid analysis (Kämpfer *et al.*, 2006).

On the basis of chemical composition and genotypic and phenotypic data presented in this paper, the isolated strains 10a^T, 11c and 12B are assigned to a novel species in a new genus, *Lysinibacillus boronitolerans* gen. nov., sp. nov., with the type strain 10a^T. The chemotaxonomic data and phylogenetic analyses (Fig. 1) also demonstrated that *Bacillus fusiformis* and *Bacillus sphaericus* should be transferred to the genus *Lysinibacillus* as *Lysinibacillus fusiformis* comb. nov. and *Lysinibacillus sphaericus* comb. nov. Data on peptidoglycan composition and polar lipids of the next-closest phylogenetically related species, *B. massiliensis* and *B. odyseeyi*, are not available; therefore, inclusion of these species in the genus *Lysinibacillus* is not recognized here. *B. silvestris* contains peptidoglycans with L-lysine and D-glutamate and its major polar lipids are phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine

Table 1. Characteristics that differentiate the novel strains (*Lysinibacillus boronitolerans*) from closely related type strains

Strains: 1, strains 10a^T, 11c and 12B (*Lysinibacillus boronitolerans* gen. nov., sp. nov.) (data from this study); 2, *B. fusiformis* DSM 2898^T (*Lysinibacillus fusiformis*) (data from Priest *et al.*, 1988); 3, *B. sphaericus* IAM 13420^T (*Lysinibacillus sphaericus*) (Claus & Berkeley, 1986); 4, *B. odysseyi* ATCC PTA-4993^T (La Duc *et al.*, 2004); 5, *B. massiliensis* CIP 108446^T (Glazunova *et al.*, 2006); 6, *B. silvestris* DSM 12223^T (Rheims *et al.*, 1999); 7, *B. pycnus* JCM 11075^T (Nakamura *et al.*, 2002); 8, *B. neidei* JCM 11075^T (Nakamura *et al.*, 2002); 9, *B. arenosi* LMG 22166^T (Heyrman *et al.*, 2005); 10, *B. arvi* LMG 22165^T (Heyrman *et al.*, 2005). All type strains produced positive results for motility, growth without NaCl and catalase activity and negative results for H₂S production and indole production. ND, No data available; v, variable results; +, positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5	6	7	8	9	10
Cell size (µm) (length × diameter)	3.0–5.0 × 0.8–1.5	ND	1.5–5.0 × 0.6–1.0	4.0–5.0 × 1.0	1.5–4.0 × 0.3–0.5	0.9–2.0 × 0.5–0.7	3.0–5.0 × 1.0–1.5	3.0–5.0 × 1.0	3.0–8.0 × 0.8–1.0	3.0–8.0 × 0.8–1.0
Spore shape and position*	R/O, T, B	R, C/T, B	R, T, B	R, T, B	ND	R, T, B	R, B	R, B	R, T, B	R, T, B
Growth temperature range (°C) (optimum)	16–45 (35–37)	17–40	10–40	25–42 (30–35)	25–45 (30–37)	10–40 (20–30)	5–45 (28–30)	5–45 (28–30)	(20–30)	(20–30)
pH range (optimum)	5.5–9.5 (7.0–8.0)	6.0–9.5	6.0–9.5	6.0–10.0 (7.0)	ND	ND	ND	ND	(7.0–9.0)	(7.0–9.0)
Growth in 7% (w/v) NaCl	–	+	–	–	–	–	–	ND	–	–
Boron tolerance (mM)	150	60†	75†	ND	ND	60†	ND	ND	ND	ND
Voges–Proskauer test	+	–	–	ND	+	–	ND	ND	–	–
Nitrate reduction	–	–	–	–	ND	–	–	–	+	–
Oxidase	+	+	+	–	+	–	ND	ND	–	–
L-Arginine dihydrolase	+	ND	ND	–	+	–	ND	ND	–	–
L-Lysine and L-ornithine decarboxylase	–	ND	ND	+	+	ND	ND	ND	–	–
Tryptophan deaminase	+	ND	– ^{a‡}	– ^a	+	– ^a	– ^a	– ^a	ND	ND
Hydrolysis of:										
Urea	+	+	–	–	+	+ ^a	– ^a	+ ^a	–	+
Gelatin	–	+	+	–	–	–	+ ^a	+ ^a	+W	–
Aesculin	+W	–	–	ND	ND	–	ND	ND	–	–
Acid production from										
Sucrose	–	V	–	–	–	+	ND	ND	–	–
D-Xylose	+W	–	–	–	–	–	ND	ND	–	–
N-Acetyl-D-glucosamine	+	ND	ND	–	ND	–	ND	ND	–	W
Resistance to (µg ml ^{–1}):										
Chloramphenicol (8)	–	+	+W	ND	–	ND	–	–	ND	ND
Erythromycin (1)	–	+	+W	ND	ND	ND	–	–	ND	ND
Rifampicin (0.25)	–	–	+W	ND	–	ND	ND	ND	ND	ND
Streptomycin (8)	–	+W	+	ND	–	ND	–	–	ND	ND
Tetracycline (2)	–	–	+	ND	–	ND	–	–	ND	ND
Oxidation of:										
Pyruvate	+	+ ^b	+ ^b	+	+	+ ^a	+	–	ND	ND
α-Hydroxybutyrate	+	– ^b	– ^b	+	–	– ^a	– ^a	ND	ND	ND
β-Hydroxybutyrate	–	– ^b	– ^b	+	–	+ ^a	+	–	ND	ND

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10
L-Alanine	+	^b	^b	+	—	^a	—	—	ND	ND
Glycyl L-glutamate	+	^b	^b	+	—	^a	—	+	ND	ND
Adenosine	+	^b	^b	—	—	^a	^a	+	ND	ND
2'-Deoxyadenosine	+	^b	^b	+	—	^a	—	+	ND	ND
Inosine	+	^b	^b	+	—	^a	—	+	ND	ND
AMP	+	^b	^b	+	—	^a	—	+	ND	ND
UMP	+	^b	^b	+	—	^a	—	+	ND	ND
16S rRNA gene sequence similarity with strain 10a ^T (%)†	(100)	97.2	96.9	96.1	95.0	94.5	93.1	93.4	93.6	93.6

*B, Bulging; C, central; O, oval or slightly oval; R, round; T, terminal.

†Data from this study.

‡Data from other studies as follows: ^a, Glazunova *et al.* (2006); ^b, La Duc *et al.* (2004).

together with minor amounts of phosphatidylserine and an unknown phospholipid. This chemical composition differentiates *B. silvestris* from members of the novel genus *Lysinibacillus*.

Description of *Lysinibacillus* gen. nov

Lysinibacillus (Ly.si'ni.ba.cil'lus. N.L. n. *lysinum* lysine; L. masc. n. *bacillus* a small staff or rod; N.L. masc. n. *Lysinibacillus* lysine bacillus, referring to the presence of the Lys–Asp type of peptidoglycan in the cell wall).

Motile, rod-shaped cells that produce ellipsoidal or spherical endospores which lie terminally in a swollen sporangium. Oxidase and catalase tests are positive, whereas the production of indole and H₂S, nitrate reduction and β -galactosidase (ONPG) tests are negative. Major cellular fatty acid is iso-C_{15:0}. Cell-wall peptidoglycan contains lysine and aspartic acid as the diagnostic amino acids, representing the cell wall peptidoglycan type A4 α (Lys–Asp). The dominant respiratory lipoquinone system is MK-7. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and ninhydrin-positive phosphoglycolipid. The G + C content is 35–38 mol%. The type species is *Lysinibacillus boronitolerans*.

Description of *Lysinibacillus boronitolerans* sp. nov.

Lysinibacillus boronitolerans (bo.ro'ni.to'le.rans. N.L. n. *boron -onis* boron; L. part. adj. *tolerans* tolerating; N.L. part. adj. *boronitolerans* boron-tolerating).

In addition to the characteristics described for the genus, the species has the following features. Cells are 3.0–5.0 μ m long and 0.8–1.5 μ m in diameter. Colonies are circular with entire margins, flat/umbonate elevation, opaque and butyrous in texture and 2–3 mm in diameter after 2 days on NA (pH 7.0) plates at 37 °C. Temperature for growth is 16–45 °C, with optimum growth at 37 °C; there is no growth at $\geq$ 50 °C and little growth at 16 °C after several days. Growth is observed at pH 5.5–9.5, with optimum growth at pH 7.0–8.0 (most rapid initial growth at pH 7.5) but no growth at pH 5.0. Tolerates 0–100 mM boron in agar media, with optimum growth in the absence of boron and some growth at 150 mM boron after 2 days. NaCl is tolerated up to 5 % (w/v), indicating that it is moderately halotolerant. Growth is seen on marine agar 2216, TSA and NA with or without addition of boron or NaCl. Acid is produced from *N*-acetyl-D-glucosamine, D-xylose (weak) and aesculin (weak) (API 50CH); acid is not produced from carbohydrates (API 20E). Positive for Voges–Proskauer test, urease, L-arginine dihydrolase, tryptophan deaminase and citrate utilization and negative for hydrolysis of gelatin and L-lysine and L-ornithine decarboxylases. Oxidizes the following substrates: D-alanine, glycogen, D-ribose, D-tagatose, inosine, L-alanine, L-alanyl glycine, β -cyclodextrin, 2-aminoethanol, L-histidine, L-leucine, L-ornithine, L-proline, L-threonine, acetic acid, glycyl L-glutamic acid, L-lactic acid, L-malic acid, L-glutamic acid, 2'-deoxyadenosine, L-serine, pyruvic acid,

Fig. 1. Phylogenetic tree showing the inter-relationships among strains 10a^T and 12B (*Lysinibacillus boronitolerans* gen. nov., sp. nov.) and their close relatives inferred from 16S rRNA gene sequences. Data with gaps were removed using BioEdit software during alignment for construction of the rooted tree using the neighbour-joining method contained in the PHYLIP package (Felsenstein, 2005) and the tree was plotted with NJPlot, based on a comparison of approximately 1294 nucleotides. Bootstrap values, expressed as percentages of 1000 replications, are given at branching points. The sequence of *Paenibacillus polymyxa* NCDO 1774^T was used as the outgroup. Bar, 1% sequence divergence.

methyl pyruvic acid, TMP, monomethyl succinate, UMP, propionic acid, alaninamide, adenosine, AMP, lactamide, L-asparagine, thymidine, uridine, α -hydroxybutyric acid, α -ketovaleric acid, bromosuccinic acid, *cis*-aconitic acid, citric acid, DL-lactic acid, formic acid, gluconic acid, glycyl L-aspartic acid, i-erythritol, L-aspartic acid and α -ketobutyric acid. Major cellular fatty acids are iso-C_{15:0} (32%), anteiso-C_{15:0} (21%), iso-C_{16:0} (11%), anteiso-C_{17:0} (11%), C_{16:1} ω 7c alcohol (8%), iso-C_{17:0} (6%) and iso-C_{14:0} (2%). Cell-wall peptidoglycan contains lysine, alanine, glutamic acid and aspartic acid as the diagnostic amino acids. In addition to the polar lipids given in the genus description, it also contains phosphatidylethanolamine. Strong enzyme activity is observed for α -chymotrypsin and esterase (C8), whereas weak activity is observed for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, acid phosphatase, valine arylamidase and naphthol-AS-BI-phosphohydrolase (API ZYM). The type strain is resistant to linomycin, colistin, sulfamethizol, oxolinic acid, fusidic acid and metronidazol (ATB-VET).

The G + C content of the type strain is 36.5 mol% (determined by HPLC).

Strain 10a^T (= DSM 17140^T = IAM 15262^T = ATCC BAA-1146^T) is the type strain, isolated from soil collected in the experimental area of the University of Tokyo (Yayoi campus), Japan.

Description of *Lysinibacillus fusiformis* comb. nov.

Lysinibacillus fusiformis (fu.si.for'mis. L. n. *fusus* spindle; L. n. *forma* shape, form; N.L. adj. *fusiformis* spindle-shaped).

Basonym: *Bacillus fusiformis* (ex Meyer and Gottheil 1901) Priest *et al.* 1988.

The description of the species as given by Priest *et al.* (1988) is unchanged. Cell-wall peptidoglycan contains lysine, alanine, glutamic acid and aspartic acid in the molar ratio of 1.81:1.0:0.69:0.64 as diagnostic amino acids. Cellular

Table 2. Characteristics that differentiate the genus *Lysinibacillus* gen. nov. from closely related genera

Data for reference genera were taken from Claus *et al.* (1992) (*Caryophanon* and *Sporosarcina*), Keddie & Jones (1992) (*Kurthia*) and Claus & Berkeley (1986) (*Bacillus*) unless indicated. +, Positive; –, negative; NA, data not available; *m*-DAP, *meso*-diaminopimelic acid.

Characteristic	<i>Lysinibacillus</i>	<i>Caryophanon</i>	<i>Kurthia</i>	<i>Sporosarcina</i>	<i>Bacillus</i>
Endospore formation	+	–	–	+	+
Rod-shaped cells	+	Trichome rods	+	–	+
Oxidase	+	–	NA	+	+
Nitrate reduced to nitrite	–	–	–	+	+
Cell-wall type	Lys–Asp	L–Lys–D–Glu ^{a*}	L–Lys–D–Asp	L–Lys–Gly–D–Glu ^a	<i>m</i> -DAP ^b
Menaquinone system	MK-7	MK-6	MK-7	MK-7	MK-7
Major fatty acid	iso-C _{15:0}	NA	NA	anteiso-C _{15:0} ^c	anteiso-C _{15:0} ^d
G + C content (mol%)	35–38†	44–46	36–38	40–42	32–69

*Data from other studies as follows: *a*, Stackebrandt *et al.* (1987); *b*, Schleifer & Kandler (1972); *c*, Yoon *et al.* (2001); *d*, Kämpfer *et al.* (2006).

†Data for G + C content of *B. fusiformis* from Fahmy *et al.* (1985) and *B. sphaericus* from Priest *et al.* (1988).

Table 3. Cellular fatty acid compositions of the novel strains and closely related taxa

Strains: 1, strains 10a^T, 11c and 12B (*L. boronitolerans*) (results are based on two replicates for each strain); 2, *B. fusiformis* (*L. fusiformis*) (data for three strains); 3, *B. sphaericus* (*L. sphaericus*) (three strains); 4, *B. arenosi* (three strains; data from Heyrman *et al.*, 2005); 5, *B. arvi* (two strains; Heyrman *et al.*, 2005); 6, *B. massiliensis* CIP 108446^T (Glazunova *et al.*, 2006); 7, *B. silvestris* DSM 12223^T (Rheims *et al.*, 1999). –, Not detected/not reported.

Fatty acid	1	2	3	4	5	6	7
C _{14:0}	0.4 ± 0.05	1.0 ± 0.04	0.4 ± 0.2	1.8 ± 0.4	1.9 ± 0.1	–	–
C _{15:0}	0.5 ± 0.07	1.1 ± 0.1	–	3.7 ± 0.9	2.2 ± 0.5	3.2	–
C _{16:0}	1.8 ± 0.36	3.5 ± 0.2	0.7 ± 0.4	1.5 ± 0.4	1.3 ± 0.0	1.5	–
C _{17:0}	–	–	–	–	–	–	0.9
iso-C _{14:0}	1.7 ± 0.48	2.8 ± 0.1	2.3 ± 0.3	5.2 ± 1.7	12.8 ± 0.3	3.1	2.9
iso-C _{15:0}	31.8 ± 4.63	23.9 ± 0.6	41.9 ± 0.4	46.2 ± 3.4	23.4 ± 2.1	48	44.2
anteiso-C _{15:0}	21.4 ± 2.57	17.9 ± 0.6	4.7 ± 0.2	9.8 ± 1.0	16.2 ± 1.1	15.3	5.6
iso-C _{15:1} at 5	–	–	0.5 ± 0.1	–	–	–	–
iso-C _{16:0}	11.2 ± 2.61	15.7 ± 0.3	8.2 ± 0.7	5.4 ± 2.1	15.1 ± 1.2	13.5	6.2
iso-C _{16:1}	–	–	–	–	–	–	18.7
iso-C _{17:0}	5.5 ± 1.01	4.4 ± 0.2	7.0 ± 1.8	3.4 ± 0.5	1.9 ± 0.2	3.7	5.1
anteiso-C _{17:0}	11.1 ± 3.22	9.2 ± 0.4	1.7 ± 0.4	3.1 ± 0.1	5.0 ± 0.4	5.6	3.0
anteiso-C _{17:1}	–	–	–	–	–	–	2.8
iso-C _{17:1} ω10c	1.3 ± 0.29	0.9 ± 0.02	8.3 ± 0.9	4.9 ± 0.1	1.3 ± 0.1	–	7.8*
C _{16:1} ω7c alcohol	7.6 ± 0.90	11.6 ± 0.31	18.3 ± 2.66	4.3 ± 1.2	10.7 ± 1.2	2.6	3.1*
C _{16:1} ω11c	2.7 ± 0.52	5.8 ± 0.19	3.3 ± 0.07	7.3 ± 0.8	6.1 ± 0.4	0.7	–
C _{18:1} ω9c	–	–	–	–	–	0.7	–
Summed feature 4†	2.8 ± 0.66	2.2 ± 0.03	2.2 ± 0.10	2.7 ± 0.2	2.4 ± 0.1	–	–

*Rheims *et al.* (1999) designated these fatty acids as iso-C_{17:1} and C_{16:1}, respectively, without providing information about the position of double bond and the type of isomer (*cis/trans*).

†Summed feature 4 comprises iso-C_{17:1} I and/or anteiso-C_{17:1} B.

fatty acid profile is listed in Table 3. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and ninhydrin-positive phosphoglycolipid. The type strain is DSM 2898^T (= JCM 12229^T = LMG 9816^T = ATCC 7055^T).

Description of *Lysinibacillus sphaericus* comb. nov.

Lysinibacillus sphaericus (sphae'ri.cus. L. masc. adj. *sphaericus* spherical).

Basonym: *Bacillus sphaericus* Meyer and Neide 1904.

In addition to the characteristics summarized by Claus & Berkeley (1986), the cellular fatty acid composition is added to the description of the species (Table 3). Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, ninhydrin-positive phosphoglycolipid and an unknown polar lipid. The type strain is DSM 28^T (= LMG 7134^T = JCM 2502^T = ATCC 14577^T = CCM 2120^T = NCIMB 9370^T = NCTC 10338^T).

Acknowledgements

We thank Dr A. Nozawa for technical guidance and suggestions, Mr X. Tian for help with the menaquinone analysis, Dr N. Bughio for providing valuable comments on the manuscript and the Ministry of Education, Culture, Sports, Science, and Technology of Japan for providing financial assistance to the first author.

References

- Ahmed, I., Yokota, A. & Fujiwara, T. (2007a). A novel highly boron tolerant bacterium, *Bacillus boroniphilus* sp. nov., isolated from soil, that requires boron for its growth. *Extremophiles* **11**, 217–224.
- Ahmed, I., Yokota, A. & Fujiwara, T. (2007b). *Gracilbacillus boracitolerans* sp. nov., a highly boron-tolerant and moderately halotolerant bacterium isolated from soil. *Int J Syst Evol Microbiol* **57**, 796–802.
- Ahmed, I., Yokota, A. & Fujiwara, T. (2007c). *Chimaericella boritolerans* sp. nov., a boron-tolerant and alkaliphilic bacterium of the family *Flavobacteriaceae* isolated from soil. *Int J Syst Evol Microbiol* **57**, 986–992.
- Ash, C., Farrow, J. A. E., Wallbanks, S. & Collins, M. D. (1991). Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Lett Appl Microbiol* **13**, 202–206.
- Ash, C., Priest, F. G. & Collins, M. D. (1993). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. *Antonie van Leeuwenhoek* **64**, 253–260.
- Chen, X., Schauder, S., Potier, N., Dorselaer, A. V., Pelczar, I., Bassler, B. L. & Hughson, F. M. (2002). Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**, 545–549.
- Claus, D. & Berkeley, R. C. W. (1986). Genus *Bacillus* Cohn 1872, 174^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1105–1139. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

- Claus, D. & Fritze, D. (1989).** Taxonomy of *Bacillus*. In *Bacillus* (Biotechnology Handbooks vol. 2), pp. 5–26. Edited by C. R. Harwood. New York: Plenum.
- Claus, D., Fritze, D. & Kocur, M. (1992).** Genera related to the genus *Bacillus* – *Sporolactobacillus*, *Sporosarcina*, *Planococcus*, *Filibacter* and *Caryophanon*. In *The Prokaryotes: a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd edn, vol. 2, pp. 1769–1791. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Clausen, V., Jones, J. G. & Stackebrandt, E. (1985).** 16S ribosomal RNA analysis of *Filibacter limicola* indicates a close relationship to the genus *Bacillus*. *J Gen Microbiol* **131**, 2659–2663.
- Cochran, D. G. (1995).** Toxic effects of boric acid on the German cockroach. *Experientia* **51**, 561–563.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989).** Fluorometric deoxy-ribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Fahmy, F., Flossdorf, J. & Claus, D. (1985).** The DNA base composition of the type strains of the genus *Bacillus*. *Syst Appl Microbiol* **6**, 60–65.
- Farrow, J. A. E., Wallbanks, S. & Collins, M. D. (1994).** Phylogenetic interrelationships of round-spore-forming bacilli containing cell walls based on lysine and the non-spore-forming genera *Caryophanon*, *Exiguobacterium*, *Kurthia*, and *Planococcus*. *Int J Syst Bacteriol* **44**, 74–82.
- Felsenstein, J. (2005).** PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- Glazunova, O. O., Raoult, D. & Roux, V. (2006).** *Bacillus massiliensis* sp. nov., isolated from cerebrospinal fluid. *Int J Syst Evol Microbiol* **56**, 1485–1488.
- Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. A. (1996).** *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234–239.
- Hall, T. A. (1999).** BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Heyndrickx, M., Lebbe, L., Kersters, K., De Vos, P., Forsyth, G. & Logan, N. A. (1998).** *Virgibacillus*: a new genus to accommodate *Bacillus pantothenicus* (Proom and Knight 1950). Emended description of *Virgibacillus pantothenicus*. *Int J Syst Bacteriol* **48**, 99–106.
- Heyrman, J., Rodríguez-Díaz, M., Devos, J., Felske, A., Logan, N. A. & De Vos, P. (2005).** *Bacillus arenosi* sp. nov., *Bacillus arvi* sp. nov. and *Bacillus humi* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **55**, 111–117.
- Kämpfer, P., Rosselló-Mora, R., Falsen, E., Busse, H.-J. & Tindall, B. J. (2006).** *Cohnella thermotolerans* gen. nov., sp. nov., and classification of ‘*Paenibacillus hongkongensis*’ as *Cohnella hongkongensis* sp. nov. *Int J Syst Evol Microbiol* **56**, 781–786.
- Katsivela, E., Bonse, D., Krüger, A., Strömpl, C., Livingston, A. & Ittich, R.-M. (1999).** An extractive membrane biofilm reactor for degradation of 1,3-dichloropropene in industrial wastewater. *Appl Microbiol Biotechnol* **52**, 853–862.
- Keddie, R. M. & Jones, D. (1992).** The genus *Kurthia*. In *The Prokaryotes: a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd edn, vol. 2, pp. 1654–1662. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Keddie, R. M. & Shaw, S. (1986).** Genus *Kurthia*. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1255–1258. Edited by P. H. A. Sneath, N. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Kimura, M. (1980).** A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kudo, T. (2001).** Phospholipids. In *Identification Manual of Bacteria: Molecular Genetics and Molecular Biological Methods*, pp. 135–144. Edited by K. Suzuki, A. Hiraishi & A. Yokota. Tokyo: Springer.
- La Duc, M. T., Satomi, M. & Venkateswaran, K. (2004).** *Bacillus odysseyi* sp. nov., a round-spore-forming bacillus isolated from the Mars Odyssey spacecraft. *Int J Syst Evol Microbiol* **54**, 195–201.
- Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A. & Parlett, J. H. (1984).** An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233–241.
- Nakamura, L. K., Shida, O., Takagi, H. & Komagata, K. (2002).** *Bacillus pycnus* sp. nov. and *Bacillus neidei* sp. nov., round-spored bacteria from soil. *Int J Syst Evol Microbiol* **52**, 501–505.
- Pechman, K. J., Lewis, B. J. & Woese, C. R. (1976).** Phylogenetic status of *Sporosarcina ureae*. *Int J Syst Bacteriol* **26**, 305–310.
- Priest, F. G., Goodfellow, M. & Todd, C. (1988).** A numerical classification of the genus *Bacillus*. *J Gen Microbiol* **134**, 1847–1882.
- Ranftl, H. & Kandler, O. (1970).** D-Aspartyl-L-alanin als Interpeptidbrücke im Murein von *Bacillus pasteurii* Migula. *Z Naturforsch [C]* **28**, 4–8 (in German).
- Rheims, H., Frühling, A., Schumann, P., Rohde, M. & Stackebrandt, E. (1999).** *Bacillus silvestris* sp. nov., a new member of the genus *Bacillus* that contains lysine in its cell wall. *Int J Syst Bacteriol* **49**, 795–802.
- Rowe, R. I. & Eckhert, C. D. (1999).** Boron is required for zebrafish embryogenesis. *J Exp Biol* **202**, 1649–1654.
- Rowe, R. I., Bouzan, C., Nabili, S. & Eckhert, C. D. (1998).** The response of trout and zebrafish embryos to low and high boron concentrations is U-shaped. *Biol Trace Elem Res* **66**, 261–270.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schleifer, K. H. & Kandler, O. (1972).** Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Shaw, S. & Keddie, R. M. (1983).** A numerical taxonomic study of the genus *Kurthia* with a revised description of *Kurthia zopfii* and a description of *Kurthia gibsonii* sp. nov. *Syst Appl Microbiol* **4**, 253–276.
- Shida, O., Takagi, H., Kadowaki, K. & Komagata, K. (1996).** Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. *Int J Syst Bacteriol* **46**, 939–946.
- Stackebrandt, E. & Goebel, B. M. (1994).** Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Stackebrandt, E., Ludwig, W., Weizenegger, M., Dorn, S., McGill, T. J., Fox, G. E., Woese, C. E., Schubert, W. & Schleifer, K.-H. (1987).** Comparative 16S rRNA oligonucleotide analyses and murein types of round-spore-forming bacilli and non-sporeforming relatives. *J Gen Microbiol* **133**, 2523–2529.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

- Wainø, M., Tindall, B. J., Schumann, P. & Ingvorsen, K. (1999).** *Gracilibacillus* gen. nov., with description of *Gracilibacillus halotolerans* gen. nov., sp. nov.; transfer of *Bacillus dipsosauri* to *Gracilibacillus dipsosauri* comb. nov., and *Bacillus salexigens* to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov. *Int J Syst Bacteriol* **49**, 821–831.
- Warrington, K. (1923).** The effect of boric acid and borax on the broad bean and certain other plants. *Ann Bot* **37**, 629–672.
- Wisotzkey, J. D., Jurtshuk, P., Jr, Fox, G. E., Deinhard, G. & Poralla, K. (1992).** Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. nov. *Int J Syst Bacteriol* **42**, 263–269.
- Xie, C. & Yokota, A. (2003).** Phylogenetic analysis of *Lamprospedia hyalina* based on the 16S rRNA gene sequence. *J Gen Appl Microbiol* **49**, 345–349.
- Yoon, J.-H., Weiss, N., Lee, K.-C., Lee, I.-S., Kang, K. H. & Park, Y.-H. (2001).** *Jeotgalibacillus alimentarius* gen. nov., sp. nov., a novel bacterium isolated from jeotgal with L-lysine in the cell wall, and reclassification of *Bacillus marinus* Rger 1983 as *Marinibacillus marinus* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **51**, 2087–2093.
- Yoon, J. H., Kang, K. H. & Park, Y. H. (2002).** *Lentibacillus salicampi* gen. nov., sp. nov., a moderately halophilic bacterium isolated from a salt field in Korea. *Int J Syst Evol Microbiol* **52**, 2043–2048.