Lysinibacillus parviboronicapiens sp. nov., a low-boron-containing bacterium isolated from soil

Hiroki Miwa,^{1,2} Iftikhar Ahmed,^{1,3} Akira Yokota⁴ and Toru Fujiwara^{1,2}

Correspondence

Toru Fujiwara

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

Hiroki Miwa

umiwa@mail.ecc.u-tokyo.ac.jp

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

²SORST, JST, Chiyoda-Ku, Tokyo, Japan

³National Agricultural Research Centre, Park Road, Islamabad 45500, Pakistan

⁴Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-Ku, Tokyo 113-0032, Japan

A spore-forming, Gram-positive-staining, motile, rod-shaped and low-boron-containing bacterium was isolated from soil. The strain, designated BAM-582^T, can tolerate 6 % (w/v) NaCl and 50 mM boron, but optimal growth was observed without addition of boron or NaCl. The optimum temperature and pH for growth were 30 °C (range 10–37 °C) and pH 7 (range pH 6–8). A comparative analysis of the 16S rRNA gene sequence demonstrated that the isolated strain was closely related to *Lysinibacillus fusiformis* DSM 2898^T (97.7 % similarity) and *Lysinibacillus sphaericus* IAM 13420^T (98.2 %). Levels of DNA–DNA relatedness were 33.9 % with *L. fusiformis* DSM 2898^T and 29.5 % with *L. sphaericus* DSM 28^T. The genomic DNA G+C content of the novel strain was 38.7 mol%. The major respiratory quinone was MK-7 and the major fatty acids were iso-C_{15:0} (37.4 %) and anteiso-C_{15:0} (19.0 %). Analysis of cell-wall amino acids revealed that the strain contained peptidoglycan with lysine, aspartic acid, alanine and glutamic acid, as is the case with other species of the genus *Lysinibacillus*. Based upon its distinctive peptidoglycan composition, phylogenetic and genotypic analyses and physiological characteristics, the strain BAM-582^T is concluded to represent a novel species in the genus *Lysinibacillus*, for which the name *Lysinibacillus parviboronicapiens* sp. nov. is proposed (type strain BAM-582^T = NBRC 103144^T = KCTC 13154^T).

Boron is a micronutrient required by plants (Warington, 1923) and possibly by animals (Rowe *et al.*, 1998; Rowe & Eckhert, 1999) and several marine algal flagellates (Loomis & Durst, 1992). Amongst bacteria, boron is required for the growth of heterocystous cyanobacteria (Bonilla *et al.*, 1990) and actinomycetes of the genus *Frankia* (Bolaños *et al.*, 2002). However, *Bacillus boroniphilus* is the only known species that cannot grow without boron (Ahmed *et al.*, 2007a). Although boron is known to be involved in quorum sensing (Chen *et al.*, 2002), the biological functions of boron in bacteria remain elusive. Boron also has a toxic effect on living cells above a certain threshold. Because of its toxicity, boron has long been used in the treatment of recurrent vulvovaginal candidiasis caused by some species of *Candida* and *Saccharomyces* (Swate & Weed, 1974; Otero *et al.*, 2002). Boron is also used as a

food preservative to sterilize against micro-organisms (Nielsen, 2004). Recently, *Bacillus boroniphilus*, *Gracilibacillus boracitolerans*, *Chimaericella boritolerans* and *Lysinibacillus boronitolerans* have been reported to tolerate more than 450, 450, 300 and 150 mM boron, respectively (Ahmed *et al.*, 2007a, b, c, d), whereas *Variovorax boronicumulans* shows intracellular accumulation of boron (Miwa *et al.*, 2008).

The genus *Lysinibacillus* was proposed recently following a re-examination of rRNA group 2 of the genus *Bacillus* (Ahmed *et al.*, 2007d). Strains of the genus *Lysinibacillus* contain lysine and aspartate in the peptide subunit of the cell-wall peptidoglycan as diagnostic amino acids rather than meso-diaminopimelic acid, which is characteristic of the genus *Bacillus*. The type species of the genus is *Lysinibacillus boronitolerans*, and its other members are *Lysinibacillus fusiformis* and *Lysinibacillus sphaericus*.

In the course of exploring boron-accumulating bacteria, strain BAM-582^T was isolated from soil of the Hisarcik area in Kutahya province, Turkey. This area has been reported to contain significant amounts of boron minerals naturally

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BAM-582^T is AB300598.

Details of the intracellular boron content of strain BAM-582^T and related type strains and results of 2D TLC of polar lipids of strain BAM-582^T are available as supplementary material with the online version of this paper.

(Çöl & Çöl, 2003). In this article, we report the taxonomic properties of strain BAM-582^T isolated from naturally boron-containing soil, which clusters with members of the genus *Lysinibacillus*.

Strain BAM-582^T was isolated from a homogenized soil sample diluted with sterilized PBS (pH 7.2) on tryptic soya agar (TSA; Difco) and incubated at 25 °C. Individual colonies isolated on TSA were used for the measurement of intracellular boron.

During preliminary screening for boron-accumulating isolates, intracellular boron was estimated by growing the isolates at 25 °C for 7 days in tryptic soya broth (TSB; Difco) containing 100 µM boric acid (Wako); cells were harvested by centrifugation and analysed for intracellular boric acid by the azomethine-H method (Gupta & Stewart, 1975). To determine the boron concentration more precisely, isolates were grown at 25 °C for 2 days in TSB (5 ml) containing 100 µM boric acid, harvested, washed with TSB and analysed for boron content by inductively coupled plasma-mass spectrometry (ICP-MS; Seiko Instruments Industry). The weight of the freeze-dried pellet was used to calculate the intracellular boron concentration. Strain BAM-582^T contained a smaller amount of boric acid compared with closely related members of the genus *Lysinibacillus* (Supplementary Table S1, available in IJSEM Online) and thus was subjected to further characterization.

The temperature for optimum growth of the strain was determined by incubation at various temperatures from 4 to 45 °C for 2 weeks on TSA medium (pH 7.2). The pH range for growth was determined using TSA adjusted to various pH values (pH 5.0–10.0 at intervals of 1.0 pH unit). The medium was buffered with 100 mM MES for pH 5.0, 100 mM NaH₂PO₄ for pH 6.0–8.0 and 100 mM Na₂CO₃ for pH 9.0 and 10.0 (Yumoto *et al.*, 2004). The pH was adjusted using KOH or HCl prior to sterilization. NaCl tolerance was examined using TSA plates containing 0–10 % NaCl. Boron tolerance was determined on TSA plates containing 25, 50, 100 and 150 mM boric acid.

The motility and morphology of cells were observed using phase-contrast microscopy, and spore formation was examined as described previously (Ahmed *et al.*, 2007a). Gram staining was performed according to Hucker's modified method (Cowan, 1974). Growth under anaerobic conditions was determined after 2 weeks incubation in an AnaeroPack (Mitsubishi Gas). Catalase and oxidase activities were determined as described by Yoon *et al.* (2006). API 20E and API ZYM galleries (bioMérieux) were used to examine physiological and biochemical characteristics; API 20E and API ZYM results were read after 48 h incubation at 25 °C and 4 h incubation at 37 °C, respectively. Utilization of various carbon sources was determined using API 50 strips (bioMérieux) and the Biolog GP2 MicroPlates (Biolog). The inoculated Biolog GP2 plate was incubated at 28 °C for 72 h before the results were read. Resistance to antibiotics was assessed by

an ATB-VET strip (bioMérieux). All commercial kits were used according to the manufacturers' protocols unless otherwise mentioned. The results from API 20E, API-50CH, Biolog GP2 and ATB-VET were compared with closely related species in Table 1 and additional characteristics are included in the species description.

The nearly complete 16S rRNA gene sequence of the strain was amplified by PCR as described by Katsivela *et al.* (1999). The purified PCR product was sequenced as described previously (Ahmed *et al.*, 2007a). The 16S rRNA gene sequence of strain BAM-582^T was compared with sequences of closely related species with validly published names retrieved from GenBank. Alignment was performed with CLUSTAL_X software (Thompson *et al.*, 1997). Gaps and ambiguous bases were not taken into consideration during comparison. Evolutionary distances and *K*_{nuc} values (Kimura, 1980) were calculated. A phylogenetic tree was constructed using the neighbour-joining method and maximum-parsimony method using MEGA version 3.1 (Kumar *et al.*, 2004) from alignment of 1283 bases of the 16S rRNA gene. The stability of relationships was assessed by bootstrap analysis by performing 1000 resamplings for the tree topology.

The 16S rRNA gene sequence (1477 nt) of strain BAM-582^T showed the highest similarity to the sequences of *L. fusiformis* NRRL NRS-350^T (98.2 %), *L. sphaericus* NRRL B-23268^T (97.7 %), *L. boronitolerans* 10a^T (96.6 %), *Bacillus massiliensis* 4400831^T (96.4 %), *Bacillus odyseeyi* 34hs-1^T (96.2 %) and *Bacillus silvestris* HR3-23^T (95.6 %), and lower sequence similarity (less than 96 %) to other neighbouring species. The phylogenetic tree constructed by the neighbour-joining method revealed that strain BAM-582^T clustered with the members of the genus *Lysinibacillus* at a high bootstrap value of 96 % (Fig. 1), showing greater reliability of the phylogeny. Phylogenetic analysis by the maximum-parsimony algorithm produced a similar result (not shown).

Genomic DNA of the novel strain was prepared according to the standard protocol and DNA G+C content was determined by the HPLC method of Mesbah *et al.* (1989). DNA–DNA hybridizations of the novel strain with type strains of closely related species showing 16S rRNA gene sequence similarity of more than 97 % (Stackebrandt & Goebel, 1994) were performed at 42 °C using a photo-biotin-labelled DNA probe as described by Ezaki *et al.* (1989). DNA–DNA hybridization values between strain BAM-582^T and *L. sphaericus* IAM 13420^T and *L. fusiformis* DSM 2898^T were 29.5 and 33.9 %, respectively, indicating that strain BAM-582^T represents a separate species of the genus *Lysinibacillus* (Stackebrandt & Goebel, 1994).

For whole-cell fatty acid analysis, 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; version 4.0) according to the manufacturer's instructions. Analysis of the respiratory quinone system was performed as described by Xie & Yokota

Table 1. Characteristics that differentiate strain BAM-582^T from species of the genus *Lysinibacillus*

Strains/species: 1, strain BAM-582^T; 2, *L. boronitolerans* 10a^T (unless indicated, data from Ahmed *et al.*, 2007d); 3, *L. fusiformis* (Priest *et al.*, 1988); 4, *L. sphaericus* (Claus & Berkeley, 1986). All members of the genus produced positive results for motility, growth without NaCl and catalase and negative results for H₂S production, nitrate reduction, oxidation of β -hydroxybutyrate and indole production. +, Positive or >85 % of strains positive; +w, weakly positive; –, negative; v, variable; ND, no data available.

Characteristic	1	2	3	4
Cell length (μ m)	2.0–5.0	3.0–5.0	ND	1.5–5.0
Cell diameter (μ m)	0.5–1.0	0.8–1.5	ND	0.6–1.0
Spore shape and position*	R, T, B	R/O, T, B	R, C/T, B	R, T, B
Temperature range for growth ($^{\circ}$ C)	10–37	16–45	17–40	10–40
pH range for growth	6–8	5.5–9.5	6.0–9.5	6.0–9.5
Growth in:				
NaCl (7 %, w/v)	–	–	+	–
Boron (mM)	50	150	60 ^{a†}	75 ^a
Voges–Proskauer test	+	+	–	–
Oxidase	–	+	+	+
L-Arginine dihydrolase	–	+	ND	ND
Tryptophan deaminase	+w	+	ND	–
Hydrolysis of:				
Urea	+	+	+	–
Gelatin	–	–	+	+
Aesculin	+w	+w	–	–
Acid production from:				
Sucrose	–	–	v	–
D-Xylose	–	+w	–	–
N-Acetyl-D-glucosamine	–	+	ND	ND
Resistance to (μ g ml ^{–1}):				
Chloramphenicol (8)	+	–	+	+w
Erythromycin (1)	–	–	+	+w
Rifampicin (0.25)	–	–	–	+w
Streptomycin (8)	+	–	+w	+
Tetracycline (2)	–	–	–	+
Oxidation of:				
Pyruvate	–	+	+ ^b	+ ^b
α -Hydroxybutyrate	–	+	– ^b	– ^b
L-Alanine	–	+	+ ^b	+ ^b
Glycyl L-glutamate	–	+	+ ^b	+ ^b
Adenosine	–	+	+ ^b	+ ^b
2'-Deoxyadenosine	–	+	+ ^b	+ ^b
Inosine	–	+	+ ^b	+ ^b
AMP	–	+	+ ^b	+ ^b
UMP	–	+	+ ^b	+ ^b
Presence of iso-C _{16:1}	+	–	–	–

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

†Data for the type strains taken from: a, Ahmed *et al.* (2007d); b, La Duc *et al.* (2004).

(2003). The purified cell wall was analysed for amino acids using two-dimensional TLC and HPLC (Shimadzu) as described elsewhere (Schleifer & Kandler, 1972; Groth *et al.*, 1996). Polar lipids were extracted and purified from 100 mg dried cells 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).

Major cellular fatty acids of strain BAM-582^T were iso-C_{15:0} (37.4 %), anteiso-C_{15:0} (19.0 %) and anteiso-C_{17:0} (9.6 %). This result is similar to those obtained from other members of the genus *Lysinibacillus* (Table 2); however, the presence of iso-C_{16:1} (4.9 %) distinguished the strain from other members of the genus *Lysinibacillus*. The major quinone system of strain BAM-582^T was menaquinone MK-7. Cell-wall analysis for diagnostic amino acids

Fig. 1. Neighbour-joining phylogenetic tree of the members of the genus *Lysinibacillus* and representatives of some other taxa based on 16S rRNA gene sequence comparisons. Bootstrap values of 50% or above (from 1000 replicates) are shown at nodes. The sequence of *Paenibacillus polymyxa* NCDO 1774T was used as the outgroup. Bar, 1% sequence divergence.

revealed that strain BAM-582^T contained peptidoglycan with alanine, glutamic acid, lysine and aspartic acid in a molar ratio of 1.93:1.0:0.86:0.89. This composition is similar to those obtained for *L. boronitolerans*, *L. fusiformis* and *L. sphaericus* (Ahmed *et al.*, 2007d) and represents peptidoglycan type A4 α (Lys–Asp), as described by Schleifer & Kandler (1972). Strain BAM-582^T shared a similar polar lipid profile with other species of the genus *Lysinibacillus*, consisting predominantly of diphosphatidylglycerol, phosphatidylglycerol and a ninhydrin-positive phosphoglycolipid (Supplementary Fig. S1). The ninhydrin-positive phosphoglycolipid found in strain BAM-582^T

shows identical chromatographic behaviour to one reported in *L. sphaericus*, suggesting that strain BAM-582^T is closely related to *L. sphaericus*. An unknown polar lipid (L1) detected in strain BAM-582^T is absent from the polar lipid profiles of *L. boronitolerans* and *L. fusiformis*, whereas phosphatidylethanolamine is absent from the polar lipid profile of *L. sphaericus*. These chemotaxonomic analyses further confirmed that strain BAM-582^T belongs to the genus *Lysinibacillus*.

The phylogenetic and genotypic analyses, chemotaxonomic distinctiveness and differential phenotypic properties were sufficient to categorize strain BAM-582^T as a member of the genus *Lysinibacillus*, and to differentiate BAM-582^T from other related species. Therefore, strain BAM-582^T is classified as the type strain of a novel species of the genus *Lysinibacillus*, for which the name *Lysinibacillus parviboronicapiens* sp. nov. is proposed.

Table 2. Cellular fatty acid compositions (%) of strain BAM-582^T and type strains of other species of the genus *Lysinibacillus*

Strains: 1, BAM-582^T; 2, *L. boronitolerans* 10a^T; 3, *L. fusiformis* DSM 2898^T; 4, *L. sphaericus* IAM 13420^T. Data for reference strains were taken from Ahmed *et al.* (2007d).

Fatty acid	1	2	3	4
C _{14:0}	0.9	0.4	1.0	0.4
C _{15:0}	0.4	0.5	1.1	–
C _{16:0}	4.1	1.8	3.5	0.7
iso-C _{14:0}	5.9	1.7	2.8	2.3
iso-C _{15:0}	37.4	31.8	23.9	41.9
anteiso-C _{15:0}	19	21.4	17.9	4.7
iso-C _{16:0}	5.9	11.2	15.7	8.2
iso-C _{16:1}	4.9	–	–	–
iso-C _{17:0}	7.9	5.5	4.4	7.0
anteiso-C _{17:0}	9.6	11.1	9.2	1.7
iso-C _{17:1} ω10c	0.9	1.3	0.9	8.3
C _{16:1} ω7c alcohol	4.2	7.6	11.6	18.3
C _{16:1} ω11c	4.9	2.7	5.8	3.3
Summed feature 4*	2.7	2.8	2.2	2.2

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

Description of *Lysinibacillus parviboronicapiens* sp. nov.

Lysinibacillus parviboronicapiens (par'vi.bo'ro.ni.ca'pi.ens. L. adj. *parvus* little, low; N.L. n. *boron* boron; L. part. adj. *capiens* containing; N.L. part. adj. *parviboronicapiens* containing little boron).

Cells are Gram-positive-staining rods (0.5–1.0 × 2.0–5.0 μm). Colonies on TSA are circular with entire margins, opaque and creamy and 2–5 mm in diameter after 3 days incubation at 30 °C. Temperature range for growth is 10–37 °C with optimum growth at 30 °C. Growth is observed in the range pH 6–8, with optimum growth at pH 7; no growth at pH 5. Tolerates 0–50 mM boron but grows optimally without boron supply. NaCl tolerance range is 0–6% (w/v) in TSA, indicating that it is moderately halotolerant. Anaerobic growth does not occur on TSA. Catalase activity is positive, whereas oxidase reaction is negative. Positive for Voges–Proskauer and urease tests and weakly positive for tryptophan deaminase, but negative for

ONPG, citrate utilization, arginine dihydrolase, lysine and ornithine decarboxylases and production of hydrogen sulfide and indole. Acid is produced from aesculin ferric citrate, *N*-acetylglucosamine, lactose and 5-ketogluconate, but not from the other substrates of the Biolog GP2 plate. Positive for esterase lipase (C8) and chymotrypsin, but negative for alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, esterase (C4), lipase (C4), cystine arylamidase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Resistant to lincomycin, colistin, enrofloxacin, metronidazole, fusidic acid, flumequine, oxolinic acid, streptomycin and chloramphenicol (ATB-VET strip). The G + C content of the type strain is 38.7 mol%.

Strain BAM-582^T (=NBRC 103144^T =KCTC 13154^T) is the type strain, isolated from soil collected in the Hisarcik area of Turkey.

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