Gracilibacillus boraciitolerans sp. nov., a highly boron-tolerant and moderately halotolerant bacterium isolated from soil

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A motile, Gram-positive, boron-tolerant and moderately halotolerant rod-shaped bacterium was isolated from a soil naturally high in boron minerals found in the Hisarcik area of Turkey. The novel isolate, designated T-16X^T, produced spherical or ellipsoidal endospores in a non-bulging or slightly swollen sporangium in a terminal position and survived in a medium containing up to 450 mM boron. Whereas it tolerated 11 % (w/v) NaCl, it also grew without NaCl or boron. The temperature range for growth was 16–37 °C (optimum 25–28 °C) and the pH range for growth was 6.0–10.0 (optimum pH 7.5–8.5). The DNA G + C content was 35.8 mol% and the major cellular fatty acids were iso-C_{15:0} and anteiso-C_{15:0} at 18.2 and 45.7 % of the total fatty acids, respectively. MK-7 (90 %) was the predominant respiratory quinone system and meso-diaminopimelic acid was the predominant diamino acid of the cell-wall peptidoglycan. Phylogenetic analysis of the 16S rRNA gene sequence revealed that the novel strain is closely related to the type strains of *Gracilibacillus orientalis* (96.7 % similarity), *G. halotolerans* (95.5 %) and *G. dipsosauri* (95.4 %). However, the maximum DNA hybridization value for this strain with these closely related strains was less than 26.2 %. On the basis of 16S rRNA gene sequence data and chemotaxonomic and physiological features, the organism T-16X^T (=DSM 17256^T = IAM 15263^T = ATCC BAA-1190^T) is proposed to be a member of the genus *Gracilibacillus* as the type strain of the novel species *Gracilibacillus boraciitolerans* sp. nov.

Boron, a non-metal micronutrient, is required in various amounts by different organisms and is essential for the optimum growth of plants (Warington, 1923) and some animals (Rowe *et al.*, 1998; Rowe & Eckhert, 1999). Its essentiality for bacteria has been reported recently (Ahmed *et al.*, 2007). A boron-containing quorum-sensing signal molecule is produced in several species of bacteria (Chen *et al.*, 2002). The antibiotics boromycin (Kohno *et al.*, 1996) and tartrolon A and B (Irschik *et al.*, 1995), which are synthesized by bacteria, also contain boron. Negrete-Raymond *et al.* (2003) described the catabolism of phenyl boronic acid in an *Arthrobacter nicotinovorans* strain, during

which boron is predicted to be released as orthoboric acid [B(OH)₃].

At elevated levels, boron is toxic to living cells. 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 used as a food preservative to sterilize against micro-organisms (Nielsen, 2004) and is also used as an insecticide against cockroaches (Cochran, 1995).

We screened and isolated several boron-tolerant species from a soil containing naturally high levels of boron from the Hisarcik area in the Kutahya Province of Turkey. This area has been reported to be naturally high in boron minerals (Çöl & Çöl, 2003). The recently reported species '*Bacillus boroniphilus*', isolated from this soil, can tolerate more than 450 mM boron and requires boron for growth (Ahmed *et al.*, 2007). From our samples, we characterized a novel bacterial strain, T-16X^T, that is moderately

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain T-16X^T is AB197126.

SEMs of cells of strain T-16X^T, details of boron tolerance for strain T-16X^T and related strains, parsimony and maximum-likelihood trees based on 16S rRNA gene sequences and results of TLC of polar lipids of strain T-16X^T are available as supplementary material in IJSEM Online.

Table 1. Characteristics that differentiate strain T-16X^T and closely related previously described species

Strains: 1, T-16X^T (*Gracilibacillus boracitolerans* sp. nov.); 2, *G. orientalis* CCM 7326^T (data from Carrasco *et al.*, 2006); 3, *G. dipsosauri* DSM 11125^T (unless indicated, data from Deutch, 1994; Lawson *et al.*, 1996); 4, *G. halotolerans* DSM 11805^T (unless indicated, data from Wainø *et al.*, 1999). +, Positive; –, negative; ND, no data. All strains produce spores in a terminal position.

Characteristic	1	2	3	4
Cell length (filament length) and width (µm)	1.8–5.5 (12.3) × 0.5–0.9	2.0–10 × 0.7–0.9	ND	2.0–5.0 × 0.4–0.6
Pigment	Light pink to red	Cream	White	Creamy white
Morphology	Thin rods and filaments	Rods	Thin rods and filaments	Thin rods and filaments
Spore shape*	S/E	S	S	E
NaCl range (%) (optimum)	0–11 (0.5–3.0)	1–20 (10)	0–15 (ND)	0–20 (0)
Boron tolerance (mM)	0–450	ND	0–150†	0–50†
pH range (optimum)	6.0–9.7 (7.5–8.5)	5.0–9.0 (7.5)	ND (7.5)	5–10 (7.5)
Oxidase activity	+	–	+	+
Hydrolysis of:				
Gelatin	–	+	+	+
Urea	–	–	–	+
Production of H ₂ S	–	–	–	+
Nitrate reduction	–	–	+	+
Voges–Proskauer test	+	ND	–	–
DNA G+C content (mol%)	35.8	37.1	39.4	38

*E, Ellipsoidal; S, spherical.

†Results from this study.

halotolerant and highly boron-tolerant. Based upon the data, we propose that the strain belongs to the genus *Gracilibacillus*. The genus *Gracilibacillus* was described by Wainø *et al.* (1999) and its three currently characterized species were isolated from extreme hypersaline environments.

We used previously described isolation and enrichment procedures to obtain strain T-16X^T (Ahmed *et al.*, 2007). The purified culture of the isolate was maintained on agar medium and also stored as a glycerol (35 %, w/v) stock at –80 °C. Sporangia and the sizes of cells grown on Luria–Bertani agar (pH 7.0) for 16 days were observed under phase-contrast microscopy, whereas flagella were observed using a scanning electron microscope according to a previously described procedure (Ahmed *et al.*, 2007). It was clearly evident that individuals of strain T-16X^T use a monotrichous flagellum (see Supplementary Fig. S1 available in IJSEM Online) for movement and produce endospores in a non-bulging or slightly swollen sporangium. Gram staining, performed according to Hucker's modified method (Cowan, 1974), revealed that the cells of strain T-16X^T are Gram-positive.

The toxic effects of high boric acid concentrations on living cells are well known (Nable *et al.*, 1997). The boron tolerance of strain T-16X^T was compared with that of *Escherichia coli* DH10B as a control, following the procedure described previously by Ahmed *et al.* (2007). Strain T-16X^T showed boron tolerance (Supplementary Fig. S2) because it grew in TSB medium (pH 7.4 ± 0.1) containing 450 mM boric acid

(H₃BO₃, referred to simply as boron in this paper). In similar tests, we observed that the closely related strain *Gracilibacillus halotolerans* DSM 11805^T tolerated up to 50 mM boron, *Gracilibacillus dipsosauri* DSM 11125^T up to 150 mM boron and *Paraliobacillus ryukyuensis* IAM 15001^T as much as 100 mM boron. Among recently reported boron-tolerant species (Ahmed *et al.*, 2007), '*B. boroniphilus*' strains are reported to withstand more than 450 mM boron and cannot survive in its absence. In contrast, strain T-16X^T tolerated 450 mM boron, but did not require it as an essential component for growth. Boron salts have often been used in microbial growth media (Stanier *et al.*, 1966); however, the biological functions that require boron in plants and microbes are not clear, despite the fact that boron is required for growth in some organisms.

The ranges of pH, NaCl concentration and temperature for growth of strain T-16X^T were determined as described previously (Ahmed *et al.*, 2007). Motility, catalase and oxidase tests, resistance to antibiotics and enzyme activities were also evaluated following procedures described previously (Ahmed *et al.*, 2007). Various physiological and biochemical tests were carried out using API 50 CHB and API 20E galleries (bioMérieux) according to the manufacturer's instructions. Physiological experiments using the API system, the determination of antibiotic resistance using ATB-VET and catalase and oxidase tests were repeated several times. The Biolog GP2 and GN2 characterization systems were used to determine various metabolic features according to the manufacturer's instructions.

Strain T-16X^T was moderately halotolerant (Table 1) and highly boron-tolerant (Supplementary Fig. S2). The strain withstood 11 % (w/v) NaCl and grew optimally in the presence of 1–3 % NaCl. Because it exhibited optimum growth at alkaline pH (range pH 7.5–8.5) and was able to grow at above pH 9.0, it was characterized as alkalitolerant, according to the definition of Jones *et al.* (1994). The best growth occurred on BUG medium containing 20 mM boron and 1 % (w/v) NaCl, but we also observed growth on marine agar 2216 (MA; Difco), tryptic soya agar (TSA) and nutrient agar (NA), although growth was slow and colonies were small. Strain T-16X^T shares many characteristics with members of the genus *Gracilibacillus*. The characteristics that differentiated strain T-16X^T from closely related type strains are shown in Table 1. Additional characteristics are included in the species description. In addition to its boron tolerance, the major characteristic that differentiated strain T-16X^T from closely related type strains was a positive Voges–Proskauer test.

A nearly complete 16S rRNA gene fragment (1500 nt) of strain T-16X^T was amplified by PCR according to the protocol described by Katsivela *et al.* (1999). The purified PCR product was sequenced as described previously (Ahmed *et al.*, 2007). The consensus sequence obtained using the DNASIS Pro software package (Hitachi Software Engineering) was compared with the sequences of closely related type strains retrieved from the DDBJ/EMBL database

using BLAST searches. An alignment was produced using CLUSTAL X 1.8 software (Thompson *et al.*, 1997) and edited using the BioEdit software package (Hall, 1999) to exclude ambiguous positions and gaps from the calculations. Ultimately, 1302 bp were used to calculate evolutionary distances and K_{nuc} values (Kimura, 1980) and to construct a phylogenetic tree using the neighbour-joining method (Saitou & Nei, 1987) in the PHYLIP software package (Felsenstein, 2005). The tree was plotted using NJplot software. To assess the stability of the phylogenetic tree, a bootstrap analysis (Felsenstein, 2005) was performed using 1000 resamplings of the neighbour-joining data. Phylogenetic trees were also generated using maximum-likelihood and maximum-parsimony algorithms, which generated relationships for the novel strain similar to those constructed using the neighbour-joining method (Supplementary Fig. S3). These analyses strongly support the affiliation of strain T-16X^T with the genus *Gracilibacillus*.

Based on the comparison of 16S rRNA gene sequence data, the highest similarity of strain T-16X^T was 96.7 % with *Gracilibacillus orientalis* XH-63^T, followed by 95.5 % with *G. halotolerans* NN^T, 95.4 % with *G. dipsosauri* DD1^T and 95.7 % with *Paraliobacillus ryukyuensis* O15-7^T. Strain T-16X^T was associated with ‘*Bacillus* group 1’ in the subgroup of halophilic or halotolerant organisms as described by Ash *et al.* (1991) and formed a clade with *G. orientalis* XH-63^T at a 64 % bootstrap value (Fig. 1). The phylogenetic

Fig. 1. Phylogenetic tree showing the interrelationships of strain T-16X^T and closely related previously described species inferred from 16S rRNA gene sequences. The tree was constructed using the neighbour-joining method contained in the PHYLIP software package (Felsenstein, 2005), based on a comparison of approximately 1302 bp, and plotted using NJplot. Bootstrap percentages ≥ 70 % from 1000 replications are given at branching points. *Geobacillus pallidus* DSM 3670^T is the outgroup. Bar, 1 % sequence divergence.

position of strain T-16X^T with *G. orientalis* was also supported by cellular fatty acid profiles (Table 2), in addition to other physiological and chemotaxonomic data.

Whole-cell fatty acid profiles for strain T-16X^T and *G. orientalis* CCM 7326^T were analysed from freeze-dried cell material grown in marine broth 2216 (MB; Difco) or MB plus 1.5 % NaCl for 24 h using the GC-based Microbial Identification system (MIDI) according to the manufacturer's instructions. The fatty acid profile of strain T-16X^T was composed predominantly of branched-chain fatty acids, with anteiso-C_{15:0} (45.7 %) as the major fatty acid, followed by iso-C_{15:0} (18.2 %), anteiso-C_{17:0} (16.9 %) and iso-C_{17:0} (3.2 %). Thus, it differed from *G. halotolerans*, in which iso-C_{17:0} is absent. The fatty acid profile was quite similar to those of other members of *Gracilibacillus*, especially *G. orientalis*, in that it contained the same components, but in slightly different amounts (Table 2). Its phylogenetic position (Fig. 1) was also closely associated with *G. orientalis*. *Halobacillus* species possess the unsaturated fatty acids C_{16:1}ω7*c* alcohol and C_{17:1}ω10*c*, which were absent from

the profiles of strain T-16X^T and other members of *Gracilibacillus*.

MK-7 (90 %) was determined to be the predominant respiratory quinone system in the novel strain using the method described by Xie & Yokota (2003), although MK-6 (10 %) was also detected as a minor component. Cell-wall peptidoglycans were analysed for amino acid content using two-dimensional TLC and HPLC (Shimadzu) as described elsewhere (Schleifer & Kandler, 1972; Groth *et al.*, 1996). The peptidoglycans of strain T-16X^T contained glutamic acid, *meso*-diaminopimelic acid, alanine and muramic acid in the molar ratio of 1.0:1.1:2.0:0.4 as the diagnostic amino acids, representing peptidoglycan type A1γ (directly cross-linked *meso*-diaminopimelic acid; Schleifer & Kandler, 1972). The 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). The polar lipid profile of strain T-16X^T consisted predominantly of diphosphatidylglycerol and

Table 2. Cellular fatty acid compositions of strain T-16X^T and closely related species

Values are percentages of total fatty acids; only fatty acids comprising >0.5 % of the total in at least one strain are included. Data for *G. dipsosauri* and *G. halotolerans* are from Wainø *et al.* (1999). tr, Trace amount; ND, not detected/not reported.

Fatty acid	T-16X ^T		<i>G. orientalis</i> CCM 7326 ^T	<i>G. dipsosauri</i> DSM 11125 ^T	<i>G. halotolerans</i> DSM 11805 ^T
	MB	MB + 1.5 % NaCl	MB + 1.5 % NaCl	MB	MB
Straight-chain					
C _{14:0}	0.7	0.8	0.6	tr	tr
C _{15:0}	1.9	2.0	1.0	2.8	6.6
C _{16:0}	5.3	5.6	9.8	16.4	14.9
C _{17:0}	0.5	0.4	3.3	ND	ND
C _{18:0}	0.7	0.4	5.9	tr	tr
Branched-chain					
iso-C _{14:0}	0.6	0.6	1.3	tr	4.1
iso-C _{15:0}	18.2	14.5	6.4	27.8	8.2
anteiso-C _{15:0}	45.7	50.8	39.0	29.7	40.9
iso-C _{16:0}	1.9	1.8	3.8	3.3	7.1
iso-C _{17:0}	3.2	2.0	3.9	6.8	ND
anteiso-C _{17:0}	16.9	18.4	19.3	13.3	9.9
anteiso-C _{19:0}	0.8	0.2	0.3	ND	ND
Unsaturated					
C _{16:1} ω7 <i>c</i> alcohol	ND	0.1	0.8	ND	ND
C _{16:1} ω11 <i>c</i>	0.2	0.3	2.3	tr*	3.0*
C _{18:1} ω9 <i>c</i>	1.0	0.5	0.7	ND	5.2†
Summed features‡					
3	0.6	0.5	0.5	ND	ND
5	0.9	0.6	ND	ND	ND

*This component was identified only as C_{16:1} by Wainø *et al.* (1999).

†This component was reported by Wainø *et al.* (1999) as the sum of two unidentified isomers of C_{18:1}, which were resolved as two components (2.73 and 2.45 %).

‡Summed features are groups of two or three fatty acids that cannot be resolved by GLC using the MIDI System. Summed feature 3 contained iso-C_{15:0} 2-OH and/or C_{16:1}ω7*c*. Summed feature 5 contained iso-C_{17:0} I and/or anteiso-C_{17:0} B.

phosphatidylglycerol (Supplementary Fig. S4). An unknown aminolipid (AL1) and three unknown polar lipids (L1, L2, L3) were also detected, which could not be identified with any of the specific spray reagents employed. However, no reagent for detection of glycolipids was used. The type species of the genus, *G. halotolerans*, was also diagnosed with MK-7 as the predominant respiratory quinone system, *meso*-diaminopimelic acid in cell-wall peptidoglycans of type A1 γ and the major polar lipids diphosphatidylglycerol and phosphatidylglycerol (Wainø *et al.*, 1999) and hence these characteristics of strain T-16X^T are in accordance with the traits of the genus *Gracilibacillus*; however, the lack of phosphatidylethanolamine, an unknown phospholipid and two unknown aminophospholipids reported to be present in *G. orientalis* (Carrasco *et al.*, 2006) distinguished strain T-16X^T from its phylogenetic neighbour (Fig. 1). These characteristics also fit many other bacilli. Thus, they do not indicate that the strain is a member of *Gracilibacillus*.

The DNA G + C content of strain T-16X^T was 35.8 mol% as determined using a procedure described previously (Ahmed *et al.*, 2007). The other three species of the genus *Gracilibacillus* have G + C contents in the range 37–39 mol%, which is slightly higher than that of strain T-16X^T. However, the highest 16S rRNA gene sequence similarity of strain T-16X^T occurred with members of *Gracilibacillus* (Fig. 1), and critical analysis of other chemotaxonomic data (Table 1) also suggested that strain T-16X^T belongs to the genus *Gracilibacillus*.

For DNA–DNA hybridization experiments, the genomic DNA of the novel strain and of the previously described closely related type strains was isolated using Qiagen Genomic-tip 500/G following the manufacturer's protocol, with a minor modification in which RNase T₁ was used in addition to RNase A. DNA–DNA reassociation was carried out as described by Ezaki *et al.* (1989) at 39 °C with photobiotin-labelled DNA in Nunc 96-well microplates. The DNA–DNA relatedness values of strain T-16X^T were 26.2 % with *G. orientalis* CCM 7326^T, 13.1 % with *G. halotolerans* DSM 11805^T, 15.2 % with *G. dipsosauri* DSM 11125^T and 16.2 % with *Paraliobacillus ryukyuensis* IAM 15001^T. These values were lower than the threshold of 70 % and thus indicate that strain T-16X^T represents a novel species (Stackebrandt & Goebel, 1994). The morphological, chemotaxonomic, phylogenetic and genotypic features of strain T-16X^T suggest that it belongs to the genus *Gracilibacillus*; we propose the name *Gracilibacillus boracitolerans* sp. nov.

Description of *Gracilibacillus boracitolerans* sp. nov.

Gracilibacillus boracitolerans (bo.ra'ci.i.to'le.rans. N.L. n. boracium boron; L. part. adj. tolerans tolerating; N.L. part. adj. boracitolerans boron-tolerating).

Cells are motile using a long monotrichous flagellum. Cells are Gram-positive, short rods, 2.0–4.5 μ m in length,

0.3–0.9 μ m in diameter, occurring singly, occasionally in pairs; filamentous cells also occur. Spherical endospores are produced in a non-swollen or slightly swollen sporangium in a terminal or subterminal position. Colonies are circular with entire margins, spreading but slightly convex, translucent and viscous in texture, 2–3 mm in diameter after 4 days growth on BUG agar medium (pH 7.5) at 30 °C. Younger colonies are dirty white, but become pink and then red in a few to several days. The pink or red pigment may diffuse into the agar medium after several days. Colonies are mostly light pink at high salt concentrations. Grows optimally at 25–28 °C; the temperature range for growth is 16–37 °C; no growth occurs at $\geq$ 45 °C and little growth at 16 °C after several days. The optimum pH for growth is 7.5–8.5, with a range of pH 6.0–10.0. The type strain can tolerate 0–450 mM boron, but grows optimally in the absence of boron. The NaCl tolerance range is 0–11 % (w/v), indicating that the type strain is moderately halotolerant; it can grow on MA, TSA and NA (with or without boron or NaCl). Oxidase, catalase, Voges–Proskauer and *o*-nitrophenyl β -D-galactopyranoside (ONPG) tests are positive, whereas tests for production of indole and H₂S, nitrate reduction, lysine and ornithine decarboxylases, arginine dihydrolase, tryptophan deaminase, utilization of citrate and hydrolysis of gelatin and urea are negative (API 20E). Can produce acid from L-arabinose, D-ribose, glucose, D-mannose, aesculin, D-cellobiose, D-maltose, D-lactose, D-melibiose and D-trehalose; weakly positive for acid production from D-xylose, methyl β -D-xylopyranoside, D-fructose, D-mannitol and D-sorbitol (API 50CHB). Can oxidize 3-methyl glucose, amygdalin, arbutin, D-cellobiose, dextrin, D-fructose, D-galactose, D-mannitol, D-mannose, D-melezitose, D-melibiose, D-psicose, D-raffinose, D-ribose, D-sorbitol, D-trehalose, D-xylose, gentiobiose, glycerol, lactulose, L-arabinose, maltose, maltotriose, palatinose, salicin, sucrose, turanose, α -D-glucose, α -D-lactose, methyl α -D-galactoside, methyl β -D-galactoside, methyl α -D-glucoside, methyl β -D-glucoside, DL-lactic acid, D-glucuronic acid, gluconic acid, pyruvic acid and α -ketobutyric acid (Biolog). The major cellular fatty acids are: anteiso-C_{15:0} (45.7 %), iso-C_{15:0} (18.2 %), anteiso-C_{17:0} (16.9 %), iso-C_{17:0} (3.2 %), C_{16:0} (5.3 %), C_{15:0} (1.9 %), iso-C_{16:0} (1.9 %), C_{18:1 ω 9c} (1.0 %) and traces (less than 1 %) of some other fatty acids. The cell wall contains peptidoglycan with *meso*-diaminopimelic acid as the diagnostic amino acid, representing cell-wall peptidoglycan type A1 γ . The dominant respiratory lipoquinone system is MK-7. Major polar lipids are diphosphatidylglycerol and phosphatidylglycerol. In addition, moderate to minor amounts of an unknown aminolipid and three polar lipids are detected. Strong enzyme activity is observed for alkaline phosphatase, β -galactosidase and α - and β -glucosidase, whereas weak enzyme activity is observed for α -galactosidase, esterase (C8), esterase lipase (C8) and leucine arylamidase (API ZYM). Resistant to penicillin, amoxicillin and metronidazole (ATB-VET Strip). The G + C content of the type strain is 35.8 mol% (HPLC).

Strain T-16X^T (=DSM 17256^T=IAM 15263^T=ATCC BAA-1190^T), which was isolated from soil naturally containing boron minerals from the Hisarcik area in the Kutahya Province of Turkey, is the type strain.

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