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ORIGINAL PAPER

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A novel highly boron tolerant bacterium, *Bacillus boroniphilus* sp. nov., isolated from soil, that requires boron for its growth

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Abstract Three strains of gram-positive, motile, rod-shaped and boron (B)-tolerant bacterium were isolated from naturally B containing soil of Hisarcik area in the Kutahya Province, Turkey. The strains, designated as T-14A, T-15Z^T and T-17s, produced spherical or ellipsoidal endospores in a terminal bulging sporangium. The strains required B for the growth and can tolerate more than 450 mM B. These also tolerated up to 7.0% (w/v) NaCl in the presence of 50 mM B in agar medium but grew optimally without NaCl. The temperature range for growth was 16–37°C (optimal of 30°C), whereas the pH range was 6.5–9.0 (optimal of 7.5–8.5). The DNA G + C content was 41.1–42.2 mol% and the predominant cellular fatty acid was iso-$C_{15:0}$. The major respiratory quinone system was detected as MK-7 and the diamino acid of the peptidoglycan was *meso*-diaminopimelic acid. Based on phenotypic and chemotaxonomic characteristics, phylogenetic analysis of 16S rRNA gene sequences data and DNA–DNA re-association values, we concluded that the three strains belong to a novel species of the genus *Bacillus*, the type strain of which is T-15Z^T and for which we proposed the name, *B. boroniphilus* sp. nov. (DSM 17376^T = IAM 15287^T = ATCC BAA-1204^T).

Keywords *Bacillus boroniphilus* · Boron tolerance · Boron requirement · Boron contaminated soil · Hisarcik area · Taxonomy

Abbreviations

B	Boron
Nts	Nucleotides
TSB	Tryptic soya broth
TSA	Tryptic soya agar
PBS	Phosphate buffered saline solution

Introduction

Boron (B), a non-metal micronutrient, has long been known to be essential for optimum growth of plants (Warington 1923). Some animals (Rowe et al. 1998; Rowe and Eckhert 1999) and unicellular eukaryotes (Lewin 1966) also require B but the level of requirement vary among different organisms (Bonilla et al. 1990). However, except for cyanobacteria (Mateo et al. 1986), B has not yet been reported to be essential for Bacteria. In bacteria, Anderson and Jordan (1961) provided evidence for B-stimulated nitrogen fixation in *Azotobacter*, although B was not required for the bacterial growth. Chen et al. (2002) isolated a B containing molecule that mediates quorum sensing in bacteria. The bacterial synthesized antibiotics boromycin (Kohno et al. 1996) and tartrolon A and B (Irschik et al. 1995) are also known to contain B. Negrete-Raymond et al. (2003) described phenyl boronic acid (PBA) catabolism in an *Arthrobacter nicotinovorans* strain, during which process, B is predicted to be released as orthoboric acid [$B(OH)_3$]. However, neither of the study demonstrated that bacterial growth was limited by the absence of B supply.

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On the other hand, B is toxic to living cells when present above a certain threshold. Environmental B toxicity occurs in many parts of the world (Nable et al. 1997) and B contaminated soils are difficult to ameliorate. In plants, the typical symptoms of B toxicity include necrosis of leaf tips and margins. Boron is also toxic to animals and microorganisms. Doses higher than the upper threshold of B exposure in humans have a deleterious effect to testes and reproductive functions (Çöl and Çöl 2003). Boron has long been used in the treatment of recurrent *Vulvovaginal candidiasis* caused by some species of *Candida* and *Saccharomyces* (Swate and Weed 1974; Otero at al. 2002). Due to its toxic effects for microorganisms, B has been used as a food preservative (Nielsen 2004) and also as an insecticide against cockroaches (Cochran 1995).

Substantial variation in tolerance to high B has been reported among plants species (Moody et al. 1988) and it is possible that microorganisms also vary greatly in B tolerance. Based on this hypothesis, we screened and isolated B-tolerant microorganisms from a naturally high B-containing soil of Hisarcik area in the Kutahya Province of Turkey. Organisms that grow on soils naturally high in a particular element such as B, are of great interest biologically for their ability to function under such extreme conditions and also as a source of tolerance gene(s) for other organisms. In this study, we present the first report, to our knowledge, of a novel species, *Bacillus boroniphilus* sp. nov. that requires B for its growth and can tolerate more than 450 mM B.

Materials and methods

Isolation and growth of the strains

Three B-tolerant bacterial strains (i.e. T-14AB, T-15Z^T and T-17s) were isolated from the soil of Hisarcik area, in the Kutahya province of Turkey. The soil samples (5 g) were incubated in 50 ml of phosphate-buffered saline (PBS) solution at 30°C for several days, while boric acid was added incrementally (10 mM boron per day). During incubation, the supernatant was streaked on Luria–Bertani (LB) agar (pH 7.0) plates containing different levels of H_3BO_3 up to 200 mM. The bacteria isolated on LB agar medium (pH 7.0) that contained high B concentrations were enriched to attain a purified culture of the isolates using standard procedures. The purified cultures of the isolates were maintained on tryptic soya agar (TSA) medium (Difco) and also stored in glycerol (35%, w/v) stocks at −80°C.

Boron tolerance

To demonstrate B tolerance of the novel strains in comparison with other B tolerant species, the isolated strains and the reference strain *Lysinibacillus boronitolerans* DSM 17140^T (AB199591) were grown in LB-broth containing 10 mM B, pH 7.0, until upper mid-log phase (OD_{600} 1.2) at 30°C with vigorous shaking. The cultures were serially diluted and spotted (7 µl) on LB agar medium (pH 7.0) containing different B levels up to 300 mM B. The inoculated plates were incubated at 30°C for 4 days before being photographed for results. In the second experiment, fresh cells (18–24 h old) of the novel strains harvested from BUG (Biolog) agar medium (pH 7.5) containing 20 mM B were dispersed in PBS solution that was used to innoculate 3 ml of tryptic soya broth (TSB) medium (pH 7.4 ± 0.1) with different B levels ranging from zero (control) to 450 mM B and were grown with vigorous shaking in test tubes at 30°C. *Escherichia coli* was used as a control to compare the growth curves. Milli-Q water was used to prepare PBS solution and TSB medium. OD_{600} was measured using a spectrophotometer (TAITEC, mini Photometer 518R, Tokyo, Japan) directly from tubes and OD_{600} versus time were plotted to obtain growth curves.

Microscopy

A phase-contrast microscope was used to examine endospores and size of cells, grown on nutrient-agar (Difco) with $MgSO_4$ (1.01 mM), KCl (13.4 mM), $FeSO_4$ (0.001 mM), $Ca(NO_3)_2$ (1.0 mM), H_3BO_3 (10 mM) and $MnCl_2$ (0.01 mM) at pH 7.0 for 7 days. For scanning electron microscopy, bacterial colonies grown on nutrient-agar (Difco) medium (pH 7.5) containing 20 mM B were fixed using 2.5% glutaraldehyde solution followed by sequential wash with ethanol–water mixtures, that was exchanged to 100% isoamyl acetate. Prior to SEM (JEOL Model JSM-6700F, Tokyo, Japan) observation, the sample was dried by critical point drying technique and then treated with platinum. Gram staining was performed according to Hucker's modified method. Colony morphology was observed on isolated colonies grown on nutrient-agar (Difco) medium (pH 7.0), containing 20 mM B for 10 days.

Physiological characteristics

All incubations were carried out at 30°C in all the characterization experiments unless otherwise mentioned. The strains were grown in TSB medium containing 20 mM B and 1% (w/v) NaCl with different pH values to determine the optimal pH range for the growth; with different NaCl levels (at pH 7.5) to determine the NaCl concentration range. To evaluate the temperature range for growth, the strains were grown on TSA medium with 20 mM B and 1% (w/v) NaCl, and incubated at different temperature conditions.

API 20E and API 50 CHB galleries (bioMérieux, France) were used to assess various physiological and biochemical characteristics. Oxidase test was performed using an oxidase reagent (bioMérieux, France) and repeated with an oxidase test kit (Eiken Chemicals, Tokyo,

Japan), while catalase activity was determined by bubble production in a 3% (v/v) hydrogen peroxide solution. Motility was also confirmed with M medium (bioMérieux, France) in addition to microscopy. Since mainly negative reactions were obtained with the API system (API 50CH and API 20E) for utilization of various carbon sources, we analyzed an extended array of the metabolic features of the strains using the BIOLOG GP2 and GN2 characterization system (BIOLOG) under the conditions described by the manufacturer. Resistance to antibiotics was assessed by an ATB–VET strip (bioMérieux, France), while enzyme activity was determined with an API ZYM strip (bioMérieux, France). All commercial kits were used according to the manufacturers' protocols. In API 20E, API 50 CHB, ATB-VET, API ZYM and Biolog experiments, 5 mM B was added to the inoculation medium, because the strains cannot grow without B. A negative control (with 5 mM B) was also run in the API and Biolog experiments.

16S rRNA gene sequencing and phylogenetic analysis

Nearly complete 16S rRNA gene sequences (1,503 nucleotides) of the strains were obtained after PCR amplification of the genes as described by Katsivela et al. (1999) using universal forward and reverse primers: 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1510R (5′-GGCTACCTTGTTACGA-3′). PCR products were purified with AMPure PCR purification kit (Agencourt Bioscience Corporation) and sequenced using the BigDye™ Terminator Cycle Sequencing Kits and six universal 16S rRNA gene sequencing primers (9F, 515F (5′-GTGCCAGCAGCCGCGGT-3′), 907F (5′-AAACTCAAAGGAATTGACGG-3′), 536R (5′-GTATTACCGCGGCTGCTG-3′), 926R (5′-CCGTCAATTCCTTTGAGTTT-3′), and 1510R), following the manufacturer's (Applied Biosystems) protocols, using ABI PRISM® 3730XL Genetic Analyzer. The DNASIS Pro (Hitachi Software Engineering, Tokyo, Japan) software package was used to assemble consensus sequences. The sequence data of the closely related validly published type strains used for construction of phylogenetic tree were retrieved from the DDBJ/EMBL database by BLAST searches. The alignment and editing was performed using CLUSTAL X (1.8 msw, Thompson et al. 1997) and BioEdit (Hall 1999) packages. Ambiguous positions and gaps were excluded during calculations. The unambiguously alignable data of 1,240 nucleotides (nts) were used to construct the phylogenetic tree. The evolutionary distances and K_{nuc} values (Kimura 1980) were generated using software contained in PHYLIP package (Felsenstein 2005). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) and plotted with NJ Plot software. The stability of the relationship was assessed by bootstrap analysis (Felsenstein 2005), by performing 1,000 re-samplings for the tree topology of the neighbour-joining data.

Cellular fatty acids, G + C content and chemotaxonomic analyses

For whole-cell fatty acids analysis, the strains were grown on TSA (Difco) containing 20 mM B at 30°C for 24 h and the cellular fatty acid profile was determined using the GC-based microbial identification system (MIDI) according to the manufacturer's protocol.

The genomic DNA of the strains was isolated from cells grown on plates according to the method of Marmur (1961), with the slight modification of using RNase T_1 in addition to RNase A. The subsequent phenol/chloroform extractions and ethanol precipitation were performed as described by Sambrook et al. (1989). Following digestion with P_1 nuclease, the G + C content of the extracted DNA was determined by HPLC (Shimadzu, column: Cosmosil 5C18R, Nacalai Tesc) at column temperature of 40°C and wave length 270 nm, using the mobile phase as 0.2 M ammonium phosphate:acetonitril in the ratio of 40:1 (Mesbah et al. 1989).

Respiratory quinones were analyzed as described by Xie and Yokota (2003). The purified cell wall was analyzed for amino acids using two-dimensional TLC and then HPLC (Shimadzu) as described elsewhere (Schleifer and Kandler 1972; Groth et al.1996).

DNA–DNA relatedness

For DNA–DNA hybridization experiments, QIAGEN Genomic-tips 500/G (Qiagen, Germany) were used to isolate genomic DNA of the novel strains and the closely related type strains, following the manufacturer's protocol with a minor modification in which RNase T_1 was used in addition to RNase A. DNA–DNA hybridization was performed at 42°C with photobiotin-labelled DNA using micro plates as described by Ezaki et al. (1989). DNA–DNA re-association experiments were carried out among the strains and the close relatives having 16S rRNA gene sequence similarity values of more than 97% to describe the strains as a novel species (Stackebrandt and Goebel 1994).

Results

The isolated novel strains from naturally high B containing soils of Hisarcik area demonstrated B tolerance (Fig. 1). It grew well in TSB medium (pH 7.4 ± 0.1) containing up to 450 mM B. Only slight growth on agar medium without B was observed (data not shown) that is likely to be due to the carry over B from the B containing inoculum in addition to B present in the agar-medium, while the minimal initial growth in the broth culture without added B (Fig. 1) might be due to B containing in the medium that was soon used up before growth ceased completely. To test B-tolerance of the phylogenetically closely related species, the following

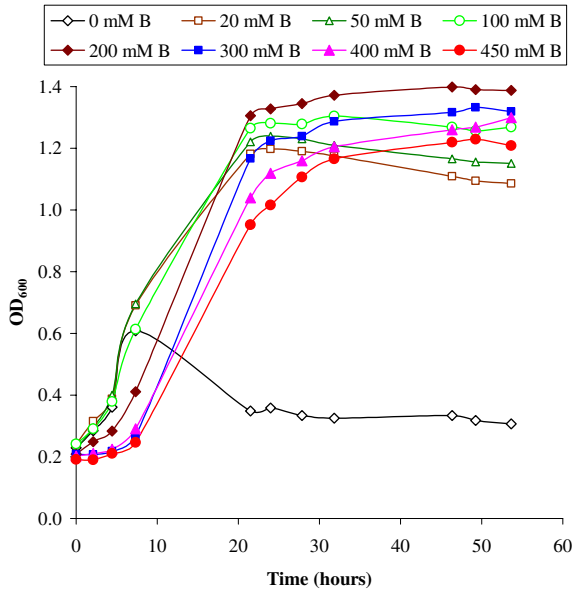

Fig. 1 Growth curves of *Bacillus boroniphilus* sp. nov. (strain T-17s) grown at different levels of B supply (mM B) in TSB medium (pH 7.4 ± 0.1)

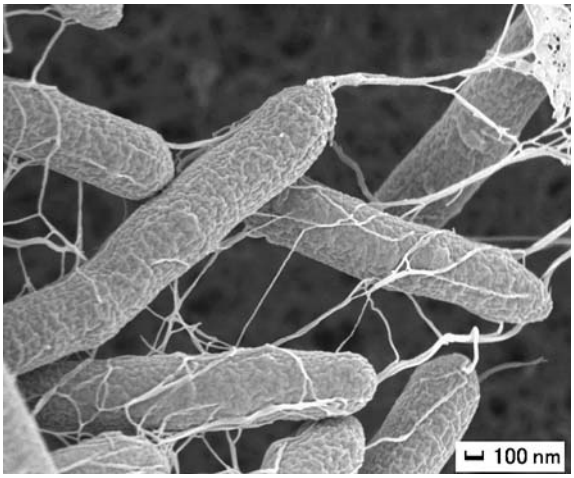

Fig. 2 Scanning electron micrograph of cells of *Bacillus boroniphilus* sp. nov. (strain T-15Z^T) grown on nutrient agar (Difco) medium (pH 7.0) with 20 mM B for 3 days at 30°C

strains were grown on media with B and found to tolerate up to (mM B, in parenthesis); *B. batavensis* DSM 15601^T (75), *B. drentensis* DSM 15600^T (50), *B. soli* DSM 15604^T (25), *B. firmis* DSM 2329^T (20) and *B. niacini* DSM 2923^T (20), while the B-tolerant reference species, *Lysinibacillus boronitolerans* DSM 17140^T only grew on plates with up to 150 mM B.

To investigate whether high osmotic conditions support the growth of the novel strains, we could not observe growth when these strains were inoculated on TSA plates containing different concentrations (20, 50 and 100 mM) of mannitol, glucose, K or Na salts without B addition, supporting that the novel strains need B for growth.

Cells of the strains occurred singly with round ends, were motile by means of peritrichous flagella (Fig. 2) and rod shaped with 1.8–5.5 μm length and 0.5–0.9 μm in diameter. Filamentous cells (up to 25 μm in length) were also observed. Oval or spherical endospores were produced terminally in a bulging sporangium. We also observed its growth on B-containing marine agar 2216 (Difco), TSA (Difco) and nutrient agar (Difco) media. The most prominent differentiating features of the strains from that of the closely related type strain, *B. jeotgali*, were their negative results for nitrate reduction and gelatin hydrolysis, B requirement for the growth, high B tolerance and the utilization of amygdalin (Table 1). The strains did not produce indole and ONPG test was also negative (API 20E).

Based on 16S rRNA gene sequences, the isolated strains differed with only one nucleotide (out of 1,503 nts) from each other, however, the highest similarity of the type strain T-15Z^T was found to be 99.8% with *B. jeotgali* (AF 221061) and 97.2% with *B. niacini* (AB 021194), followed by 97.2% with *B. soli* (AJ 542513); 97.1% with *B. firmus* (D 16268); 97.0% with *B. batavensis* (AJ 542508) and 96.9% with *B. drentensis* (AJ 542506). About 100 nts from the 5′-end and 105 nts from the 3′-end could not be used in the analysis, as the 16S rRNA gene sequence data of some reference type strains retrieved from database lacked these regions. The strains occupied a position in the phylogenetic tree with *B. jeotgali* as its close relative with 100% bootstrap value (Fig. 3), showing greater reliability of phylogenetic position of the strains. This is also in accordance with phenotypic and chemotaxonomic data.

Table 1 Characteristics that differentiate *Bacillus boroniphilus* sp. nov. and the most closely related species, *Bacillus jeotgali*

Characteristics	*Bacillus boroniphilus* sp. nov.	*Bacillus jeotgali*
Size (length × diameter, μm)	1.8–5.5 (22) × 0.5–0.9	4.0–6.0 × 0.8–1.1
Spores shape and position	E/R, T/St	E, T/St
Growth at		
pH range	6.5–9.0	
(optimum)	(7.5–8.5)	(7–8)
Temp range, °C	16–37	10–45
(optimum)	(27–30)	(30–35)
Boron (mM)	5–>450	0–75[a]
(optimum)	(100–250)	
Nitrate reduction	–	+
Gelatin hydrolysis	–	+
Utilization of amygdalin	+	–
G + C content, mol%	41.1–42.2	41.0

Bacillus jeotgali JCM10885^T data from Yoon et al (2001)
E ellipsoidal, *R* round, *T* terminal, *St* subterminal, + positive, – negative
[a]Data of this study

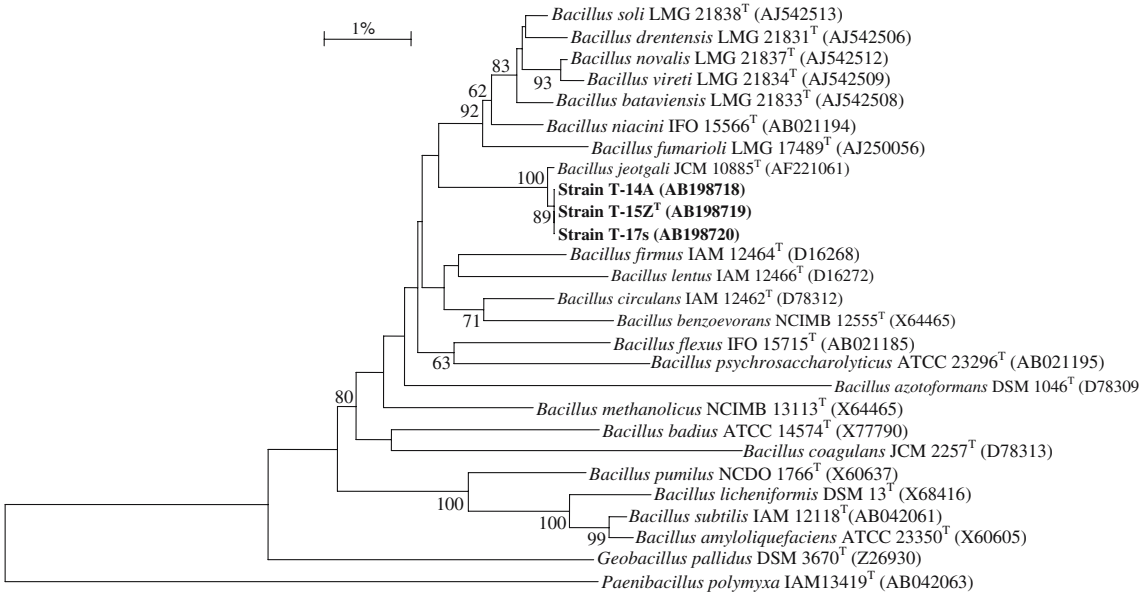

Fig. 3 Phylogenetic tree showing inter-relationship of the three strains (T-14A, T-15Z^T and T-17s) of *Bacillus boroniphilus* sp. nov. with the most closely related *Bacillus* species inferred from sequences of 16S rRNA gene. Data with gaps were removed during alignment for the construction of tree, which is rooted by using *Paenibacillus polymyxa* (AB042063) as an out group. The tree was generated using the neighbour-joining method contained in PHYLIP software package (Felsenstein 2005) based on a comparison of approximately 1,240 nts and plotted using NJ Plot software. Bootstrap values (only > 60% are shown), expressed as a percentage of 1,000 replications, are given at the branching points. The sequence of Bar, 1% sequence divergence. The accession number of each type strain is shown in *parentheses*

The strains had a cellular fatty acid profile comprised predominantly of branched chain fatty acids, with the major fatty acid being iso-$C_{15:0}$ (44.8 ± 12.8%) followed by anteiso-$C_{15:0}$ (12.5 ± 1.3%), anteiso-$C_{17:0}$ (9.3 ± 3.8%), $C_{17:1}\omega 10c$ (6.9 ± 3.7%), iso-$C_{17:0}$ (5.0 ± 0.9%) and other minor components (Table 2). The isolated strains contained MK-7 (93.1–95.3%) as the predominant respiratory quinone system, although MK-6 (4.7–6.9%) was also detected along with MK-7 as a minor component. The predominant respiratory quinone system, MK-7 is a characteristic common to *Bacillus* species (Claus and Berkeley 1986). The three strains comprised of cell wall peptidoglycan with glutamic acid, *m*-diaminopimelic acid, alanine, and muramic acid in the molar ratio of 1:1:2:0.4, respectively, as the diagnostic amino acids representing peptidoglycan type A1γ (i.e. directly cross linked *meso*-diaminopimelic acid), as described by Schleifer and Kandler (1972).

Although DNA base composition is an important indicator at the species level in general but *Bacillus* species have a wide range of G + C content as described by Priest et al. (1988) and Fahmy et al. (1985). The G + C content of the strains ranges between 41.1 and 42.2 mol% as determined by HPLC. The DNA relatedness among the isolated strains (i.e. T-14AB, T-15Z^T and T-17s) was > 99%, whereas with the validly published *Bacillus* were found to be maximum 36.4% (Table 3) with *B. jeotgali*. These values are less than the threshold of 70% to describe the strains as a novel species (Stackebrandt and Goebel 1994).

Table 2 Total cellular fatty acid (with ± SD) profile of *Bacillus boroniphilus* sp. nov. (values are average of three strains i.e.T-14AB, T-15Z^T and T-17s)

Characteristics	*Bacillus boroniphilus* sp. nov.
Straight-chain fatty acid	
$C_{14:0}$	0.64 ± 0.02
$C_{16:0}$	1.5 ± 0.5
Branched-chain fatty acid	
iso-$C_{14:0}$	0.7 ± 0.2
iso-$C_{15:0}$	44.8 ± 12.8
anteiso-$C_{15:0}$	12.5 ± 1.3
iso-$C_{16:0}$	1.6 ± 0.7
iso-$C_{17:0}$	5.0 ± 0.9
anteiso-$C_{17:0}$	9.3 ± 3.8
iso-$C_{17:1}$ $\omega 10c$	6.9 ± 3.7
Unsaturated-fatty acid	
$C_{16:1}$ $\omega 7c$ alcohol	1.5 ± 1.1
$C_{16:1}$ $\omega 11c$	3.9 ± 2.2
Summed feature	
4	6.8 ± 3.3

The summed features delineate two or more fatty acids that could not be separated by GLC with MIDI system. Summed feature 4 includes one or more of the iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B. suffix *c* indicates *cis* isomer

Discussion

Toxic effect of boric acid at high concentrations to living cells is well established phenomenon (Nable et al. 1997). In addition to toxicity tolerance against B, we also

Table 3 DNA–DNA relatedness among the strains (T-14AB, T-15Z^T and T-17s) of *Bacillus boroniphilus* sp. nov. and their most closely related *Bacillus* species

Strains	T-15Z^T = *Bacillus boroniphilus* sp. nov. DSM 17376^T
T-17s = *Bacillus boroniphilus* DSM 17377	99.5
T-14AB = *Bacillus boroniphilus*	99.2
Bacillus jeotgali JCM10885^T	36.4
Bacillus drentensis DSM 15600^T	26.8
Bacillus firmus DSM 12^T	26.5
Bacillus soli DSM 15604^T	25.3
Bacillus batavensis DSM 15601^T	24.8
Bacillus niacini DSM 2923^T	21.6

observed that the isolated strains could not grow without B (Fig. 1), suggesting that B is essential for the growth for some unknown functions. An analysis of the data depicted a decrease in growth even at 20 and 50 mM B levels after several hours but at high B levels, the growth remains constant at stationary phase with a slight increase, indicating that the strains are borophilus one (Fig. 1). The only example reported for the physiological function of B at molecular level is the case in plants where it forms esters with a *cis*-diol moiety in rhamnogalacturonan-II (RG-II) that is required for stabilization and integrity (Bolaños et al. 2004); however, RG-II has not yet been identified in bacteria, so the molecular basis for the essentiality of B in these strains is not clear at the stage.

The mechanism of high B tolerance in these strains is not known. B tolerance in plants is reported to be efflux of excess B from the cell that keeps B concentration at low level in the cell (Hayes and Reid 2004). However, these novel strains tolerate several fold of B toxicity than of plants so it is possible that these follow some other mechanism to mitigate the extreme conditions. The fact that only these novel strains require B as an essential nutrient while others do not, is not unusual because B requirement differs from species to species as was clear from an evolutionary study of the acquisition of an essential role for B in the metabolism of plants (Lovatt 1985).

The findings of B tolerance and requirement for the novel strain provide a genetic resource to identify the gene(s) responsible for the mechanism of B tolerance in bacteria because of its small genome size. Such gene(s) may be useful for cloning in other organisms especially crop species that are grown on high B soils. The characterization of B-gene(s) in *B. boroniphilus* sp. nov. may also be useful for discussing the biochemical functions of B.

Taxonomy

The three strains (T-17s, T-15Z^T, T-14A) have similar phenotypic and phylogenetic characteristics except texture of colonies. The DNA relatedness among the isolated strains (i.e. T-14AB, T-15Z^T and T-17s) was more than 99%, indicating that these isolates belong to the same species, as is further evident from 16S rRNA gene sequence similarity values, phenotypic and the chemotaxonomic data among the strains. Phylogenetic affiliation of the strains was found to be with genus *Bacillus* (Fig. 3) as is clear from the results of 16S rRNA gene sequence comparison with the *Bacillus* species. This has also been supported by chemotaxonomic data of cellular fatty acid profiles containing iso- and anteiso- types of branched fatty acids of 15:0 and 17:0 as the major entities, MK-7 as being the dominant menaquinones system (Claus and Berkeley 1986) and A1γ type of peptidoglycan (Schleifer and Kandler 1972). The DNA–DNA relatedness values of the isolated strains with the validly published *Bacillus* species were found to be maximum 36.4% (Table 3). These values are less than the threshold of 70% to describe the strains as a novel species (Stackebrandt and Goebel 1994). The different morphological, chemotaxonomical, phylogenetic and genotypic features of the strains suggested as a novel species in the genus *Bacillus* and we proposed the name, *B. boroniphilus* sp. nov. and the description of the species follows:

Description of *B. boroniphilus* sp. nov

Bacillus boroniphilus (bo.ro.ni'phi.lus. N.L. n. *borononis*, boron; Gr. adj. *philos* loving; N.L. masc. Adj. *boroniphilus*, boron-loving).

Cells are motile by means of peritrichous flagella, gram-positive, rod-shaped, 1.8–5.5 μm in length and 0.5–0.9 μm in diameter, occurring mostly single but some times in pairs and filamentous with a length of 25 μm. Ellipsoidal or spherical endospores grow terminally in a swollen sporangium. Colonies are circular with entire margins, convex with shiny and smooth surface, opaque and viscous in texture and grow 2–3 mm in diameter after 10 days at 30°C on nutrient-agar (Difco) medium (pH 7.0), containing 20 mM B. The optimum temperature for growth is 30°C; there is no growth observed at 45°C and slight growth at 16°C after several days. It can grow at a pH range of 6.5–9.0 with optimum pH of 7.5–8.5. This species can tolerate more than 450 mM B in TSB medium with optimum growth at 100–200 mM B, whereas NaCl salt tolerance range is up to 7.0% (w/v, in TSA medium along with 20 mM B), indicating that it is moderately halotolerant. It can grow in the presence of 20 mM B on Marine agar 2216 (Difco), TSA and NA. Oxidase and catalase tests are positive and H_2S gas is produced; whereas Voges–Proskauer test, indole production and NO_3 reduction, gelatin and urea hydrolysis, L-arginine dihydrolase, tryptophane deaminase, L-lysine and L-ornithine decarboxylase and β-galactosidase are negative. Aesculin is hydrolyzed in API 50 CH galleries. In Biolog system, all the three strains were positive for the following substrates: 3-methyl glucose, acetic acid, D,L-lactic acid, D-fructose, D-mannose, D-trehalose, gentiobiose, gluconic acid,

glycerol, glycogen, L-alanine, L-asparagine, L-aspartic acid, L-lactic acid, L-proline, L-serine, maltose, maltotriose, α-D-glucose, and α-ketovaleric acid; whereas at least two strains were positive for these substrates: 2′-deoxy adenosine, adenosine, adenosine-5′-monophosphate, cellobiose, dextrin, D-gluconic acid, D-psicose, D-ribose, glycyl-L-aspartic acid, glycyl-L-glutamic acid, inosine, L-alanyl-glycine, L-glutamic acid, L-leucine, L-threonine, methyl pyruvate, mono-methyl succinate, *N*-acetyl-D-glucosamine, pyruvic acid, thymidine, thymidine-5′-monophosphate, tween-40, uridine, uridine-5′-monophosphate, α-cyclodextrin, α-hydroxybutyric acid, α-ketoglutaric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, D-galacturonic acid, α-D-flucose, and propionic acid. Major cellular fatty acids are: iso-$C_{15:0}$; anteiso-$C_{15:0}$; summed feature 4; anteiso-$C_{17:0}$; iso-$C_{17:0}$; $C_{17:1}$ $\omega 10c$; and $C_{16:1}$ $\omega 11c$. MK-7 was detected as major respiratory quinone system and *m*-diaminopimelic acid as a diagnostic amino acid in peptidoglycan of cell wall. Strong enzyme activity is observed for α-glucosidase, leucine arylamidase and alkaline phosphatase; moderate for α-chemotrypsin, esterase lipase (C8) and valine arylamidase whereas weak activity is observed for α-mannosidase, β-glucosidase, trypsin and cystine arylamidase (API ZYM Strip). The strains are resistant to, metronidazol, sulfamethizol, but weakly sensitive to linomycin, penicillin, oxacillin and rifampicin (ATB-VET Strip). The G + C content of the type strain is 42.2 mol% (as determined by HPLC).

Strain T-15Z^T (DSM 17376^T = IAM 15287^T = ATCC BAA-1204^T) is a type strain of novel species, *B. boroniphilus* sp. nov., isolated from soil sampled from the Hisarcik area that is naturally high in B mineral contents, in the Kutahya Province of Turkey.

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