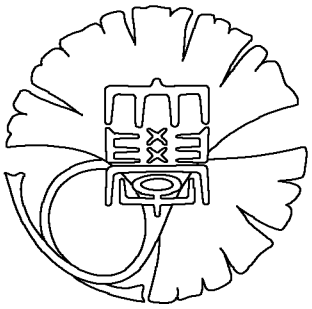
博士論文

Identification and Characterization of Boron-requiring and Boron-tolerant Bacteria: a New Frontier in Extremophiles

高ホウ素耐性および高ホウ素要求性細菌の同定と解析
- 極限微生物の新しい領域 -



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TOKYO-JAPAN
2007

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A thesis submitted as a requirement
for the degree of

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IN

ENVIRONMENTAL MICROBIOLOGY AND BIOTECHNOLOGY

BIOTECHNOLOGY RESEARCH CENTRE
THE UNIVERSITY OF TOKYO
TOKYO-JAPAN
2007

Dedicated

to

My loving Father

and

Mother (late)

Who always pray

To see the bud of

Their wishes

Bloom into a Flower

"It is certified that the contents and form of the thesis entitled “Identification and Characterization of Boron-requiring and Boron-tolerant Bacteria: a New Frontier in Extremophiles” submitted by Mr. IFTIKHAR AHMED, Registration No. 39-47111, have been found satisfactory for the award of degree of the Doctorate of Philosophy."

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(IFTIKHAR AHMED)

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CHAPTER I

EXECUTIVE SUMMARY

論文の内容の要旨

応用生命工学　　専攻
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論文題目

Identification and Characterization of Boron-requiring and Boron-tolerant Bacteria: a New Frontier in Extremophiles
(高ホウ素耐性および高ホウ素要求性細菌の同定と解析-極限微生物の新しい領域ー)

Several classes of microorganisms have been recognized as extremophiles inhabiting in an extreme environments of heat or cold, pH, salinity, pressure, and even radiation. These extremophiles, having many interesting biological secrets, provide a novel source of discoveries in applied and basic sciences. We studied a new frontier in extremophiles, i.e., microorganisms tolerating toxic levels of boron.

Boron (B), a non-metal micronutrient, has been known to be essential for plants since long. Some animals and unicellular eukaryotes also require B but the level of requirement differs among various organisms. 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 and B contaminated soils are difficult to ameliorate. Due to its toxic effects for microorganisms, B has been used as a food preservative and also as an insecticide against cockroaches. The problem of both B deficiency and toxicity becomes two-folds because of its limit being narrow for deficiency and toxicity.

Substantial variation in tolerance to high B has been reported among plant species and it is possible that microorganisms also differ greatly in B tolerance. Based on this hypothesis, we isolated and identified several B-tolerant bacterial strains from normal soil of greenhouse area (The University of Tokyo) and a naturally high B-containing soil of Hisarcik area (Kutahya Province, 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 related gene(s). This thesis reports isolation, identification and characterization of highly B-tolerant bacteria, especially this represents the first report, to our knowledge, of the novel species of bacteria that requires B for its growth and that can tolerate more than 450 mM B. Additionally, a B toxicity tolerance mechanism has also been demonstrated.

Boron-tolerant species

The strains isolated from normal greenhouse soil were found to tolerate only up to 150 mM B; however, those isolated from the soil of Turkey that is naturally high in B-containing minerals could grow at 450 mM B, an extremely high concentration of B. Phylogenetic analyses based on comparative 16S rRNA gene sequence data demonstrated that these B-tolerant strains belong to six genera; *Rhodococcus, Arthrobacter, Chimaereicella, Gracilibacillus, Lysinibacillus* and *Bacillus.* The *Bacillus* species were found to be the highest B-tolerant and could grow in TSB medium containing more than 450 mM B (Figure 1.1); followed by *Gracilibacillus, Chimaereicella, Lysinibacillus, Rhodococcus* and *Arthrobacter* tolerating B concentration of 450, 300, 150, 100 and 80 mM B, respectively. It is also observed that the neighboring species in their respective clusters could not tolerate toxic levels of B as much as these novel taxa.

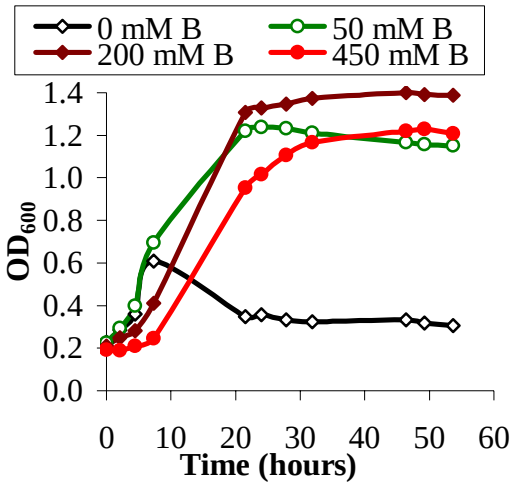

Figure 1.1. Growth curves of *Bacillus boroniphilus* sp. nov. grown at different levels of B supply in TSB medium.

Based upon phylogenetic analyses, DNA-DNA homology, phenotypic and chemotaxonomic data, the isolated strains belong to the genera; *Bacillus* (three strains), *Gracilibacillus* (one strains), and *Chimaereicella* (one strains), have been characterized as *B. boroniphilus* sp. nov., *G. boraciitolerans* sp. nov., and *C. boritolerans* sp. nov. (Ahmed *et al.*, 2007a, b, & c), respectively. These species were isolated from soil of Hisarcik area (Turkey) that was reported to be naturally high in B-minerals.

So far a peptidoglycan consisting of Lys–Asp has not been reported for any other endospore–forming species of the *Bacillus* group 2 except the novel B-tolerant strains of *L. boronitolerans* sp. nov. (three strains) along with the neighboring clade consisting of *B. fusiformis* and *B. sphaericus.* Therefore, on the basis of Lys-Asp in the cell wall peptidoglycans and other chemotaxonomic data, this B-tolerant species has been assigned to a new genus, *Lysinibacillus boronitolerans* gen. nov. sp. nov (Ahmed *et al.*, 2007d). This chemotaxonomic data and phylogenetic analyses also demonstrated that *B. fusiformis* and *B. sphaericus* should be transferred to the genus *Lysinibacillus* as *L. fusiformis* comb. nov. and *L. sphaericus* comb. nov., respectively.

The other eight B-tolerant strains belonging to the genera; *Rhodococcus* (six strains) and *Arthrobacter* (two strains) have the highest 16S rRNA gene sequence similarity (> 99%) with the closed relatives in their respective clusters and therefore, have not been included in the characterization studies.

Boron-requiring species

Although essentiality of B has been established for plants and for some animals, however, B has not yet been reported to be essential for *Bacteria*, except for cyanobacteria. During the course of these studies, a bacterial species, *B. boroniphilus* sp. nov. has been identified with the unique feature that it requires boron for its growth (Figure 1.1). The fact that the novel strain requires 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. Our data also showed 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.

Mechanism of boron tolerance

Studies of tolerance to B toxicity in the B-tolerant strains demonstrated that these B-tolerant strains maintained significantly lower B concentration in the cells in comparison to sensitive strain i.e. *Bacillus subtilis* as a control (Figure 1.2). Critical analysis of data showed an apparent negative correlation between the protoplasmic boron concentration and the degree of tolerance to a high external boron concentration (Figure 1.2). Time course B uptake studies of B-tolerant strain showed rapid uptake of B but was able to maintain steady-state cellular soluble B concentration four-folds less than the sensitive strain (Figure 1.3). Further analysis showed a decrease in cellular soluble B concentration, suggesting that efflux and/or exclusion of B is a mechanism of tolerance against high external concentration of B in prokaryotes.

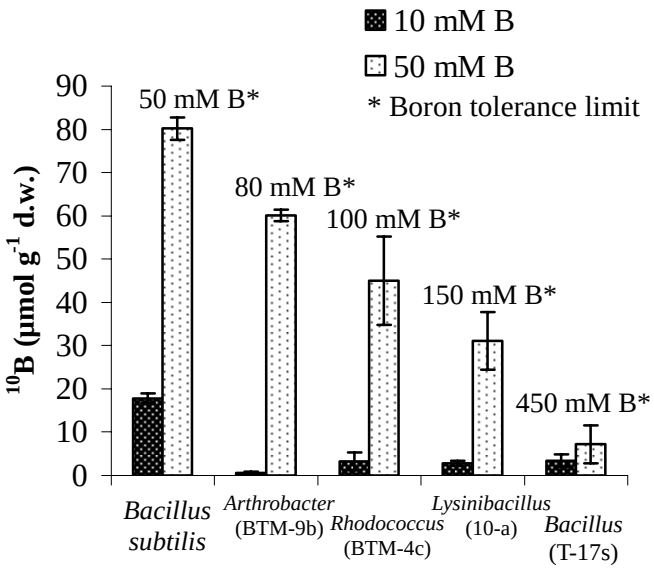

Figure 1.2. Uptake of boron in cells at two levels (10 and 50 mM) of boron supply for one hour. Data are means with error bars (± SD) for four independent replications. d.w., dry weight.

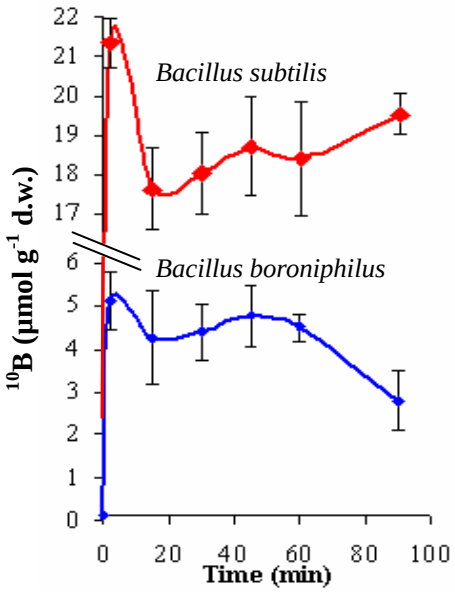

Figure 1.3. Time-course of B influx and efflux in the cells at 10 mM of boron supply. Data are means with error bars (± SD) for four independent replications. d.w., dry weight.

General conclusions

Bacillus boroniphilus could not grow without B (Figure 1.1), suggesting that B is essential for the growth, for some unknown functions. It is hypothesized that B may be needed as a structural component of cell wall as 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; it is also clear from our data that when B in the medium is used up during the initial few hours of growth, the bacterium could not survive, supporting our conclusion. Although B salts have often been included in microbial growth media, but B functions are not yet clear in prokaryotes. Identification of B-requiring strain will provide enormous information to understand the biochemistry of B in living cells.

Finally, the findings of B tolerance and essentiality 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.

CHAPTER II

BACKGROUND

Microorganisms that grow in hostile or extreme environments are currently a popular and an emerging subject for study/scientific research. These microorganisms have been recognized as 'extremophiles', which inhabit environments with extreme levels of salinity, pH, pressure, heat, cold, and even radiation. These extremophiles, having many interesting biological secrets, provide a novel source of scientific discoveries in the basic and applied sciences. In this thesis, we reported boron (B)-toxicity tolerance of bacteria as a new frontier in extremophiles.

Boron functions

Boron (B) has long been known as an essential micronutrient for 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 varies 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*.

Many authors reported biological functions of B in plants and animals, wherein its main role is suggested not only in keeping cell wall structure but also in maintaining membrane function, and supporting metabolic activities (Dordas & Brown, 2000; Dannel *et al.*, 2002; Brown *et al.,* 2002; Läuchli, 2002; Luis *et al.*, 2004). Although B salts have often been used in microbial growth media (Stainer *et al*., 1966) but the biological functions of B in microbes are not clear. Recently, Chen *et al*. (2002) isolated a B containing molecule that mediates quorum sensing in bacteria. Anderson and Jordan (1961) also provided evidence for B-stimulated nitrogen fixation in *Azotobacter*, although B was not required for the bacterial growth. 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, 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.

Boron toxicity

The fascination with B is reflected by its unique feature of a narrow window between sufficiency and toxicity. Boron may become potentially toxic to living cells when present at above a certain threshold. This problem occurs in many parts of the world (Nable *et al.,* 1997), and areas include the dry lands of South Australia (Cartwright *et al.* 1984, 1986), the Middle East (Racikocitch *et al.*, 1961), the west coast of Malaysia (Shorrocks, 1964), valleys along southern coast of Peru (Masson, 1967), the Andes foothills in northern Chile (Caceres *et al.*, 1992), solonchaks and solonetz soils of India (Takkar, 1982), rendzinas in Israel (Ravikocitch *et al.*, 1961), and major $B_2O_7^{-2}$ deposits at Searles, Lake California (Chesworth, 1991). The major source of the excess B into environment is through soil weathering processes and B-containing waste waters (Howe, 1998; Coughlin, 1998).

Excess B significantly suppresses the growth of living organisms and reduces crop yields and qualities. Necrosis of leaf tips and margins include the typical symptoms of B toxicity. B is also toxic to animals and microorganisms. High doses of B above a certain threshold in humans have toxic effects on testis and reproductive functions where as low doses result in positive effects on healing of wounds and cerebral functions (Çöl and Çöl, 2003). The tolerable intake of B in humans is reported to be 0.4 mg Kg^{-1} body weight per day where as the safe B concentration in irrigation water for plants is $\leq$ 0.3 mg L^{-1} for sensitive plants; $\leq$ 2 mg L^{-1} for semi-tolerant plants and 2-4 mg L^{-1} for tolerant plants (Nielsen, 2004). Due to sterilizing effects for microorganisms, B has been included as a food preservative (Nielsen, 1997), in addition to its use as an insecticide against cockroaches (Cochran, 1995).

Considerable genetic variation among plant species in response to high B has been identified (Moody *et al.,* 1988) and it is possible that microorganisms also vary greatly in B tolerance. However, to my knowledge, there is no report on B toxicity tolerance and/or requirement in bacteria. The 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.

The aim of this study

To understand the mechanisms of B requirement and toxicity tolerance for successful management of B in agriculture requires carrying out concrete basic and strategic research. The identification of new microorganisms has been increasing exponentially to explore the biotechnological potential that will provide a way to understand basic biochemical pathways in life sciences such as under extreme conditions of elemental toxicity.

In the present work, we attempted to isolate and identify B-requiring (chapter 2) and B-tolerant (Chapter 2-6) microorganisms from a naturally high B-containing soil of Hisarcik area (Kutahya Province, Turkey) and a normal soil of greenhouse area (The University of Tokyo, Japan). Some of them were taxonomically characterized as a novel species. This thesis presents the first report, to our knowledge, on the identification and characterization of such novel species of bacteria that requires B for its growth and that can tolerate more than 450 mM B. Additionally, a B toxicity tolerance mechanism has also been suggested (Chapter 6).

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CHAPTER III

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

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 ºC (optimal of 30 ºC), 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 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, *Bacillus boroniphilus* sp. nov. (DSM 17376^{T} = IAM 15287^{T} = ATCC BAA-1204^{T}).

Abbreviations

B: boron, nts: nucleotides, TSB: tryptic soy broth, TSA: tryptic soy agar, PBS: phosphate buffered saline solution

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* The DDBJ/EMBL/GenBank accession numbers for the 16S rRNA gene sequence of the strains T-14AB, T-15Z^{T} and T- 17s are AB198718, AB198719 and AB198720, respectively.

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 & 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 & 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.

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 testis and reproductive functions (Çöl & Çöl, 2003). 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). 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 30ºC 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 boron up to 200 mM. The bacteria isolated on LB agar medium (pH 7.0) that contained high boron concentrations were enriched to attain a purified culture of the isolates using standard procedures. The purified cultures of the isolates were maintained on tryptic soy agar (TSA) medium (Difco) and also stored in glycerol (35 %, w/v) stocks at –80 ºC.

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 ºC 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 ºC 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 soy 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 ºC. *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} vs. 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 B) 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 (Cowan, 1974). 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 ºC 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 sequencing and phylogenetic analysis

Nearly complete 16S rRNA gene sequences (1503 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^{TM} Terminator Cycle Sequencing Kits and six universal 16S rRNA 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 were 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 1240 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 & Nei, 1987) and plotted with NJ Plot software. The stability of the relationship was assessed by bootstrap analysis (Felsenstein, 2005), by performing 1000 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 ºC 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 40ºC and wave length 270 nm, using the mobile phase as 0.2 M ammonium phosphate: acetonitrile in the ratio of 40:1

(Mesbah *et al.,* 1989).

Respiratory quinones were analyzed as described by Xie & Yokota (2003). The purified cell wall was analyzed for amino acids using two-dimensional TLC and then HPLC (Shimadzu) as described elsewhere (Schleifer & 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 species, 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 sequence similarity values of more than 97 % to describe the strains as a novel species (Stackebrandt & Goebel, 1994).

RESULTS

The isolated novel strains from naturally high B containing soils of Hisarcik area demonstrated B tolerance (Figure 3.1). 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 (Figure 3.2) 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 (Figure 3.1) 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 strains were grown on media with B and found to tolerate up to (mM B, in parenthesis); *Bacillus 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.

Although we investigated 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.

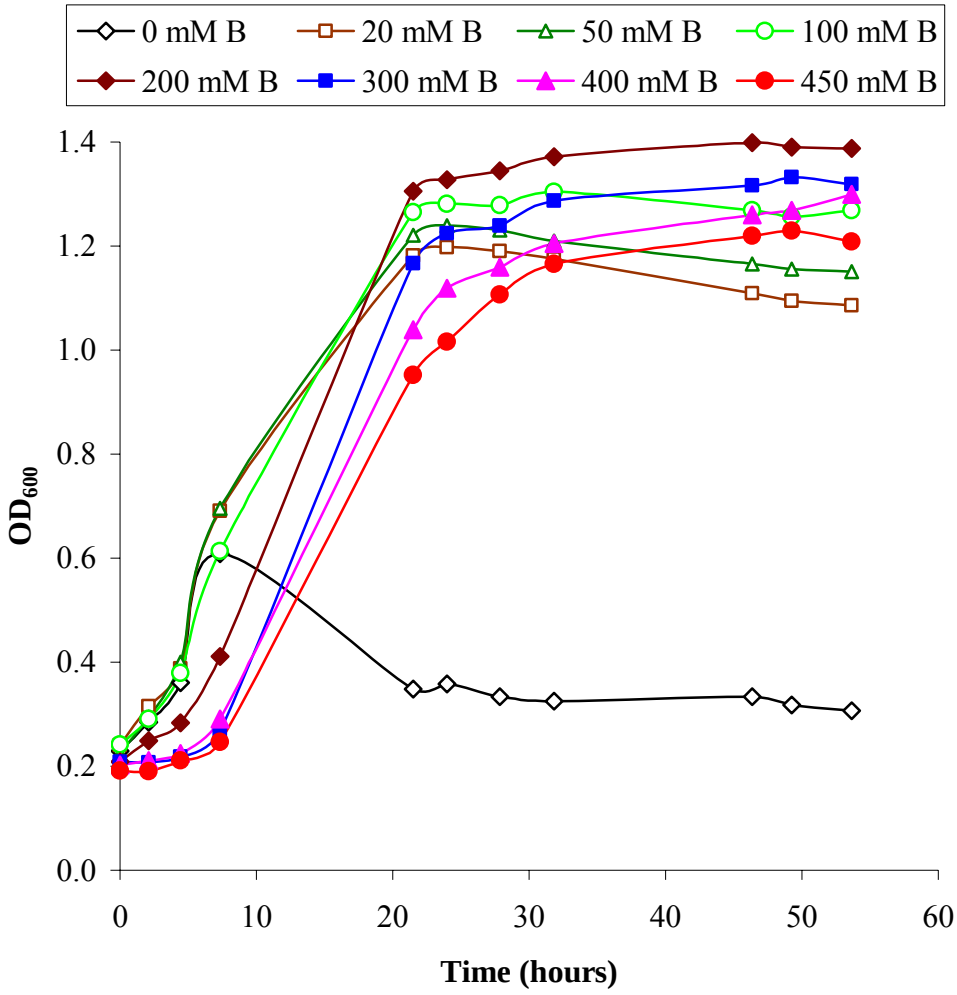

Figure 3.1.

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).

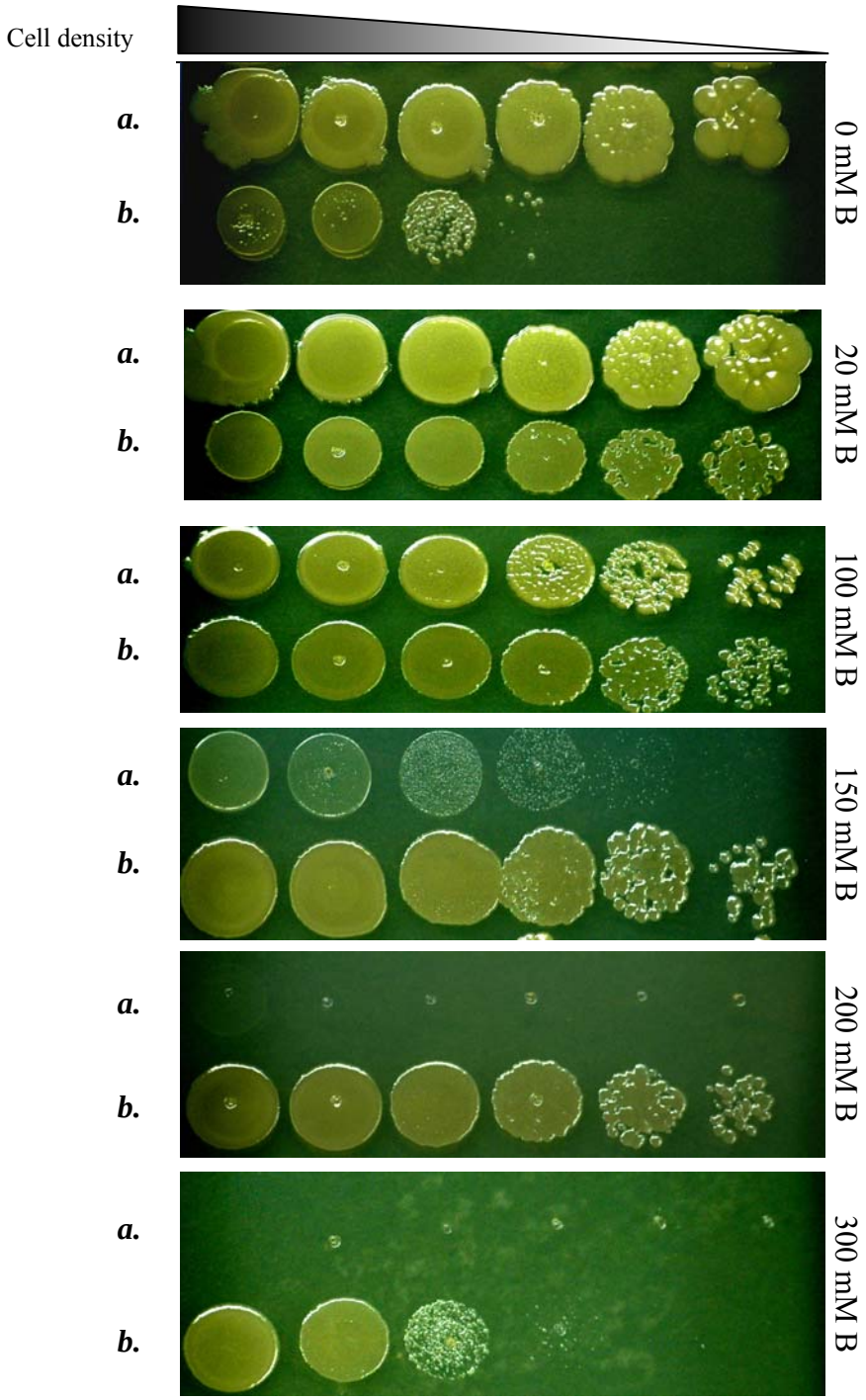

Figure 3.2.

Growth of *Lysinibacillus boronitolerans* (control, a) and the strain T-17s^{T} (b) on LB-agar medium (pH 7.0) with different concentrations of B. The strains grown in LB broth (pH 7.0) up to upper mid-log phase (OD_{600} ~1.0) at 30 ºC, were serially diluted and spotted on agar medium (pH 7.0) containing different B levels. The plates were incubated for 4 days at 30 ºC.

Cells of the strains occurred singly with round ends, were motile by means of peritrichous flagella (Figure 3.3) 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, *Bacillus 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 3.1). The strains did not produce indole and ONPG test was also negative (API 20E).

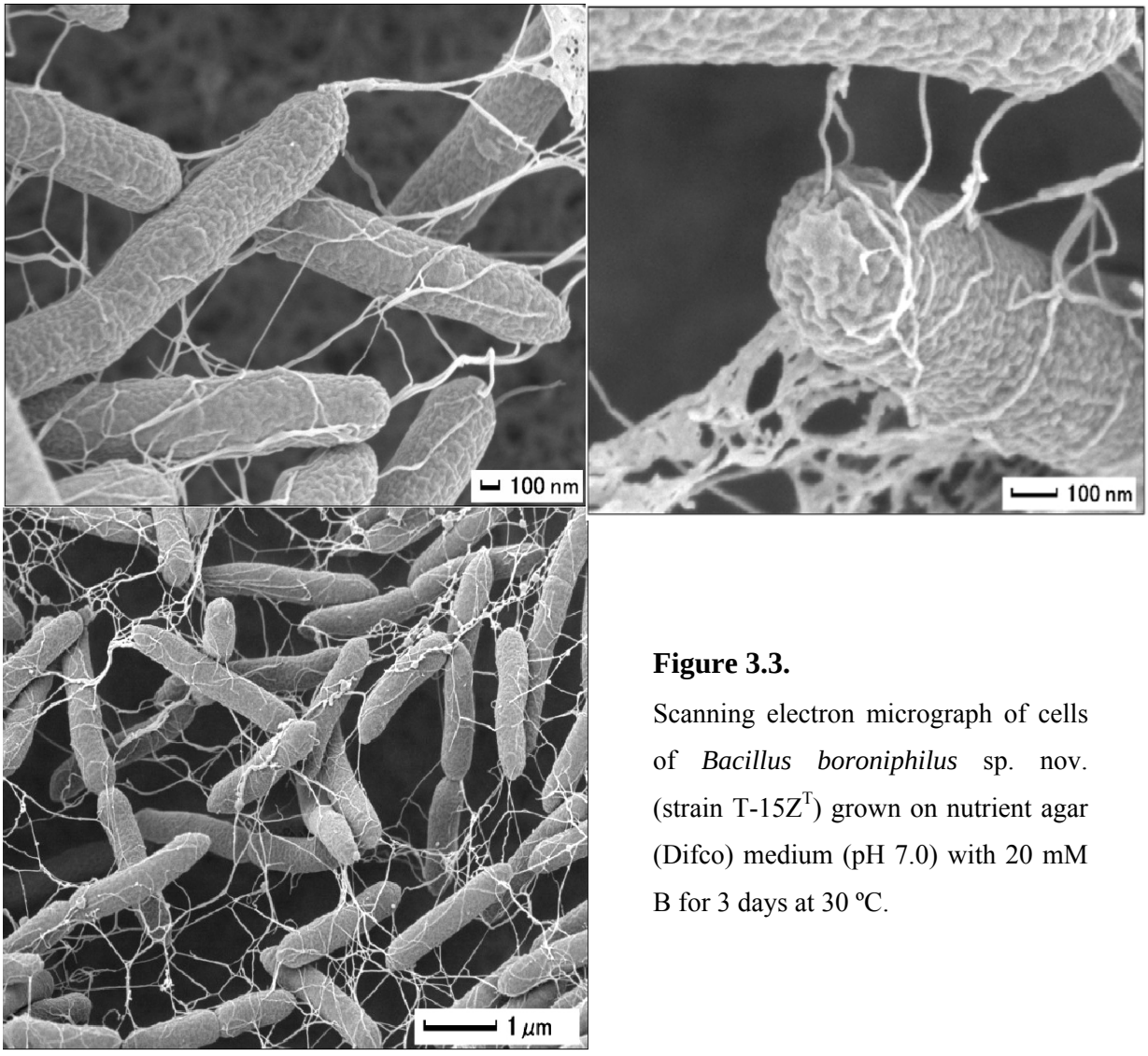

Figure 3.3.

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 ºC.

TABLE 3.1. Characteristics that differentiate *Bacillus boroniphilus* sp. nov. and the most closely related species.

Bacillus niacini DSM 2923^T data from Nagel and Andreesen (1991); *B. batavensis* DSM 15601^T, *B. soli* DSM 15604^T, *B. drentesis* DSM 15600^T and *B. novalis* DSM 15603^T data from Heyrman et al. (2004); *B. firmus* DSM 2329^T data from Heyrman et al. (2004), Claus and Berkeley (1986) and Gordon et al. (1977).

Symbols: E ellipsoidal, R round, T terminal, St subterminal, C central, Pc paracentral, + positive, (+) 75–85 % positive or w weakly positive, (-) 16–25 % positive or weakly positive, - negative, v variable among strains, nd data not determined.

Characteristics	***B. boroniphilus***	***B. jeotgali***	***B. niacini***	***B. batavensis***	***B. soli***	***B. firmus***	***B. drentensis***	***B. novalis***
Size (length X diameter, µm)	1.8–5.5 (22) X 0.5–0.9	4.0–6.0 X 0.8–1.1	3.0–5.6 X 0.9–1.4	0.7–1.2	0.6–1.2	1.2–4.0 X 0.6–0.9	0.6–1.2	0.6–1.2
Motility	+	+	–	+	+	v	+	+
Growth at:								
pH range (optimum)	6.5–9.0 (7.5–8.5)	(7–8)	(7–8)	4.5–9.5 (7–8)	4.5–9.5 (7–8)	nd	5.5–10.0 (7–8)	4–10 (7–9)
Temp. range, ºC (optimum)	16–37 (27–30)	10–45 (30–35)	10–40	~ –50 (30)	~ – 45 (30)	10–40	~ –50 (30)	~ –50 (30–40)
Boron (mM) (optimum)	5– >450 (100–250)	0-75*	0–20*	0–75*	0–25*	0–20*	0–50*	nd
H_2S production	+	nd	nd	–	–	nd	–	–
Indole production	–	–	+	–	–	–	–	–
Nitrate reduction	–	+	+	+	+	+/w	v	+/w
ONPG	–	nd	nd	+	–	–	+	–
Gelatin hydrolysis	–	+	+	+	+	v	–	+
Utilization of:								
Amygdalin	+	–	+	v	–	nd	v	v
Citrate	–	nd	+	–	–	nd	–	–
Glycogen	+	+	–	–	+	v	–	–
D-Galactose	–	–	+	+	V	nd	nd	w
L-Arabinose	–	–	+	–	–	–	–	–
D-Melezitose	–	–	+	+	–	–	v/w	–
G + C content, mol %	41–42	41	37–39	39–40	40–40	36-47	39–39	40–41

* Data of this study.

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 3.2). 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 (Figure 3.4). The predominant respiratory quinone system, MK-7 is a characteristic common to *Bacillus* species (Claus & 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 & Kandler (1972).

TABLE 3.2. 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-$C_{17:1}$ ω10*c*	6.9 ± 3.7
Unsaturated- fatty acid:	
$C_{16:1}$ ω 7 *c* alcohol	1.5 ± 1.1
$C_{16:1}$ ω 11 *c*	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.

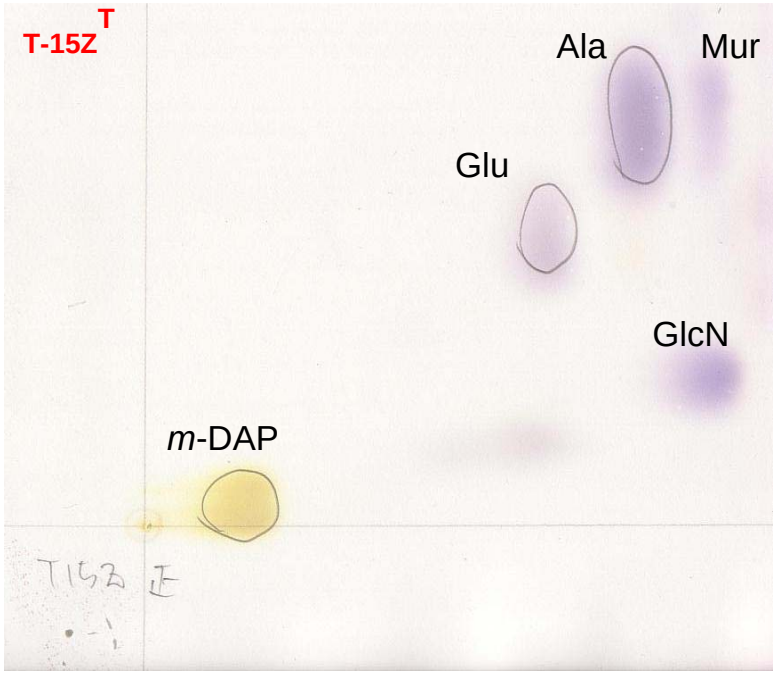

Figure 3.4.

Cell wall peptidoglycans amino acids of the strain T-15Z^T as analyzed by using two-dimensional thin-layer chromatography (2-D TLC).

Based on 16S rRNA sequences, the isolated strains differed with only one nucleotide (out of 1500 nts) from each other, however, the highest similarity of the type strain T-15Z^T was found to be 99.8 % with *Bacillus 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 sequence data of some reference type strains retrieved from database lacked these regions. The strains occupied a position in the phylogenetic tree with *Bacillus jeotgali* as its close relative with 100 % bootstrap value (Figure 3.5), showing greater reliability of phylogenetic position of the strains. This is also in accordance with phenotypic and chemotaxonomic data.

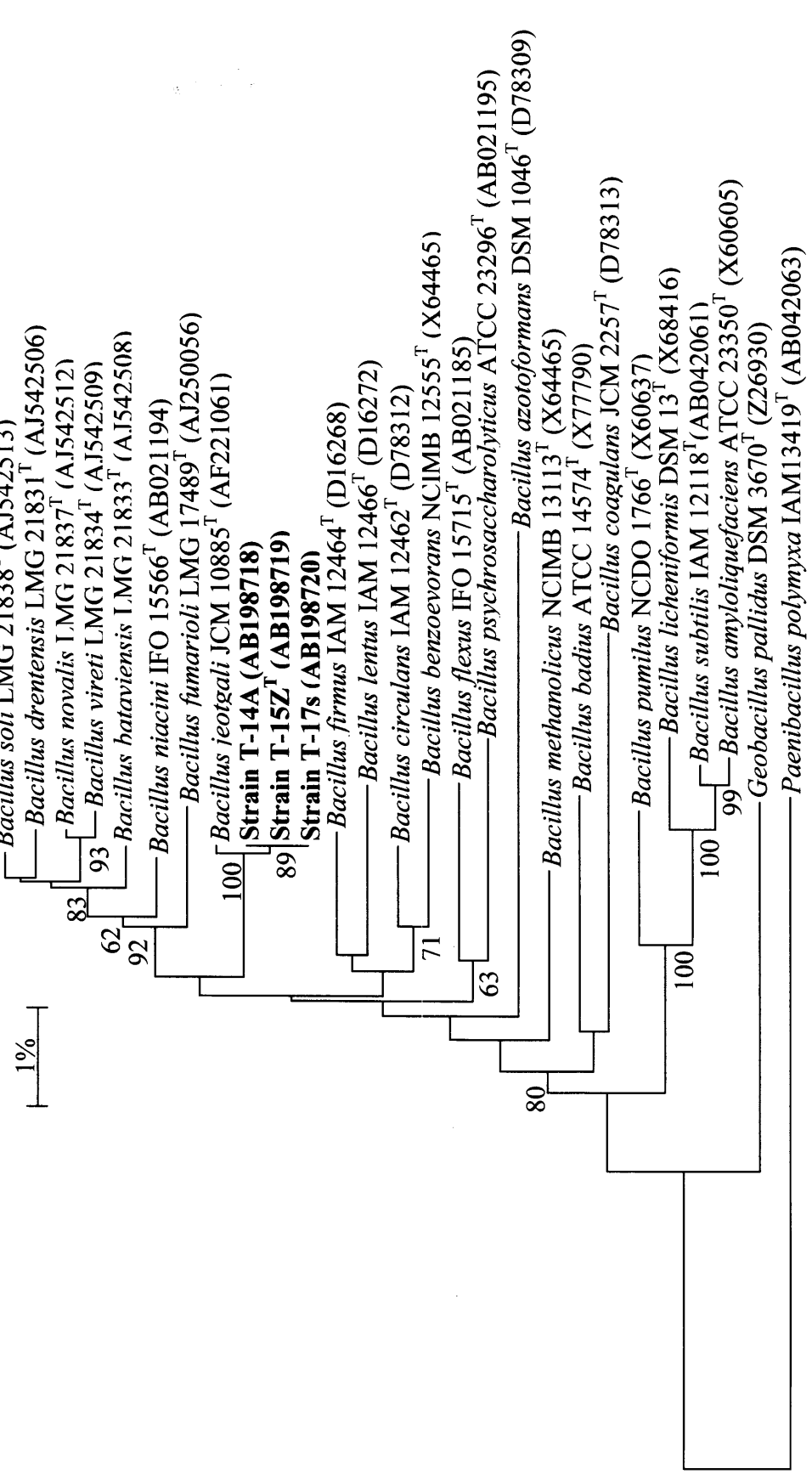

Figure 3.5.

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 type 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 1240 bps and plotted using with NJ Plot software. Bootstrap values (only >60% are shown), expressed as a percentage of 1000 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.

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 41.1–42.2 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 type species were found to be maximum 36.4 % (Table 3.3) with *Bacillus jeotgali*. These values are less than the threshold of 70 % to describe the strains as a novel species (Stackebrandt and Goebel 1994).

TABLE 3.3. DNA-DNA relatedness among the strains (T-14AB, T-15Z^T and T-17s) of *Bacillus boroniphilus* sp. nov. and their most closely related type 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

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 observed that the isolated strains could not grow without B (Figure 3.1), 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 (Figure 3.1). 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 essentially 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 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 *Bacillus 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 sequence similarity values, phenotypic and the chemotaxonomic data among the strains. Phylogenetic affiliation of the strains was found to be with genus *Bacillus* (Figure 3.5) 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 & Berkeley, 1986) and A1γ type of peptidoglycan (Schleifer & Kandler, 1972). The DNA-DNA relatedness values of the isolated strains with the validly published type species were found to be maximum 36.4 % (Table 3.3). These values are less than the threshold of 70 % to describe the strains as a novel species (Stackebrandt & Goebel, 1994). The different morphological, chemo-taxonomical, phylogenetic and genotypic features of the strains suggested as a novel species in the genus *Bacillus* and we proposed the name, *Bacillus boroniphilus* sp. nov. and the description of the species follows:

Description of *Bacillus boroniphilus* sp. nov.

Bacillus boroniphilus (bo.ro.ni'phi.lus. N.L. n. *boron –onis,* 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 ºC on nutrient-agar (Difco) medium (pH 7.0), containing 20 mM B. The optimum temperature for growth is 30 ºC; there is no growth observed at 45 ºC and slight growth at 16 ºC 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, tryptophan 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 quinine 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, *Bacillus 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.

Acknowledgments

We gratefully extend our thanks to Dr. Cavit Çöl for his kind help in collecting soil samples, to Dr. Atsushi Yamazoe for providing help and technical guidance in Biolog experiment, to Dr. Marcelino Suzuki (CBL, University of Maryland) and Professor Dr. Richard Bell (Murdoch University, Perth, Australia) for critically reading/editing this chapter and their valuable comments.

CHAPTER IV

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

ABSTRACT

We isolated a motile, Gram-positive, boron-tolerant, and moderately halotolerant rod-shaped bacterium 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 at 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 16–37ºC (optimal of 25–28ºC), and the pH range for growth was 6.0–10.0 (optimal of 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 *m*-diaminopimelic acid was the predominant cell wall peptidoglycan. Phylogenetic analysis of the 16S rRNA gene sequence revealed that this novel strain is closely related to *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 taxa 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.

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* The DDBJ/EMBL/GenBank accession number for the 16S rRNA gene sequence of strain T-16X^T (DSM 17256^T = IAM 15263^T = ATCC BAA-1190^T) is AB197126.

INTRODUCTION

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 quite recently (Ahmed *et al.*, 2007a). 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 (PBA) 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 microorganisms (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. The recently reported species *Bacillus boroniphilus* can tolerate more than 450 mM boron and requires boron for growth (Ahmed *et al.*, 2007a). From our samples, we characterized a novel species of bacterium that is moderately halotolerant and highly boron-tolerant. Based upon the data, we propose that the strain belongs to the genus *Gracilibacillus* as *Gracilibacillus boraciitolerans* sp. nov. The genus *Gracilibacillus* was described by Wainø *et al.* (1999) and its three currently characterized species were isolated from extreme hypersaline environments.

Isolation and morphology

Strain T-16X^T was isolated in soil sampled 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). We used previously described isolation and enrichment procedures (Ahmed *et al*., 2007a). The purified culture of the isolate was maintained on agar medium and also stored in glycerol (35%, w/v) stock at –80ºC. Sporangia and the sizes of cells grown on Luria–Bertani (LB) agar (pH 7.0) for 16 days were observed under phase-contrast microscopy, whereas flagella were observed using a scanning electron microscope (SEM) according to a previously described procedure (Ahmed *et al.*, 2007a). It was clearly evident that individuals of strain T-16X^T use a monotrichous flagellum (Figure 4.1) for movement

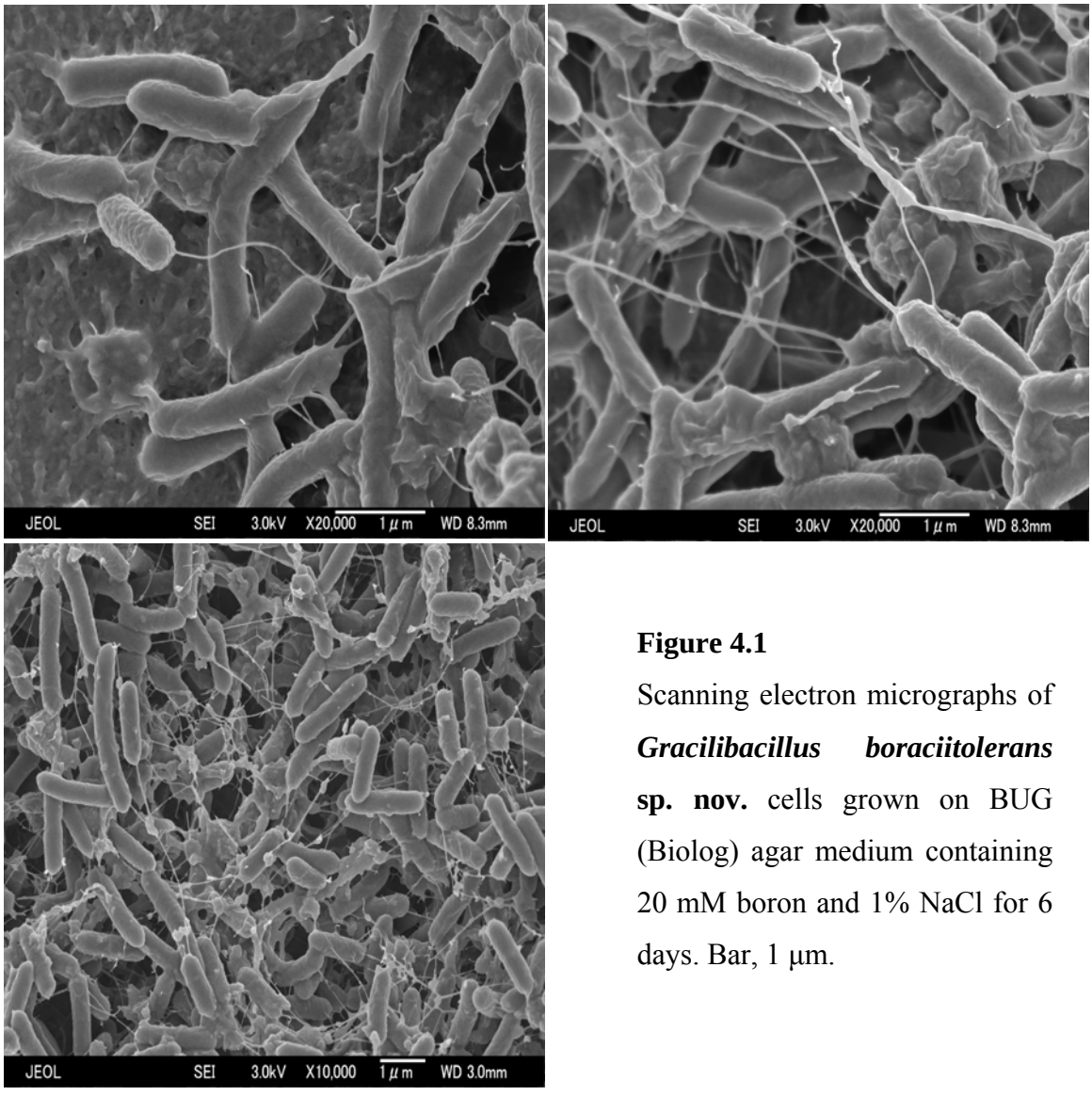

Figure 4.1
Scanning electron micrographs of ***Gracilibacillus boraciitolerans* sp. nov.** cells grown on BUG (Biolog) agar medium containing 20 mM boron and 1% NaCl for 6 days. Bar, 1 μm.

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.

Boron-tolerance assay

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 to that of *Escherichia coli* as a control, following the procedure described previously by Ahmed *et al.* (2007a). The isolated novel strain T-16X^T showed boron tolerance (Figure 4.2) because it grew in TSB medium (pH 7.4 ± 0.1) containing 450 mM boric acid (H_3BO_3, referred to as boron in this manuscript). In similar tests, we observed that the closely related type strain *G. halotolerans* tolerated up to 50 mM boron, *G. dipsosauri* up to 150 mM boron, and *Paraliobacillus ryukyuensis* as much as 100 mM boron. Among recently reported boron-tolerant species

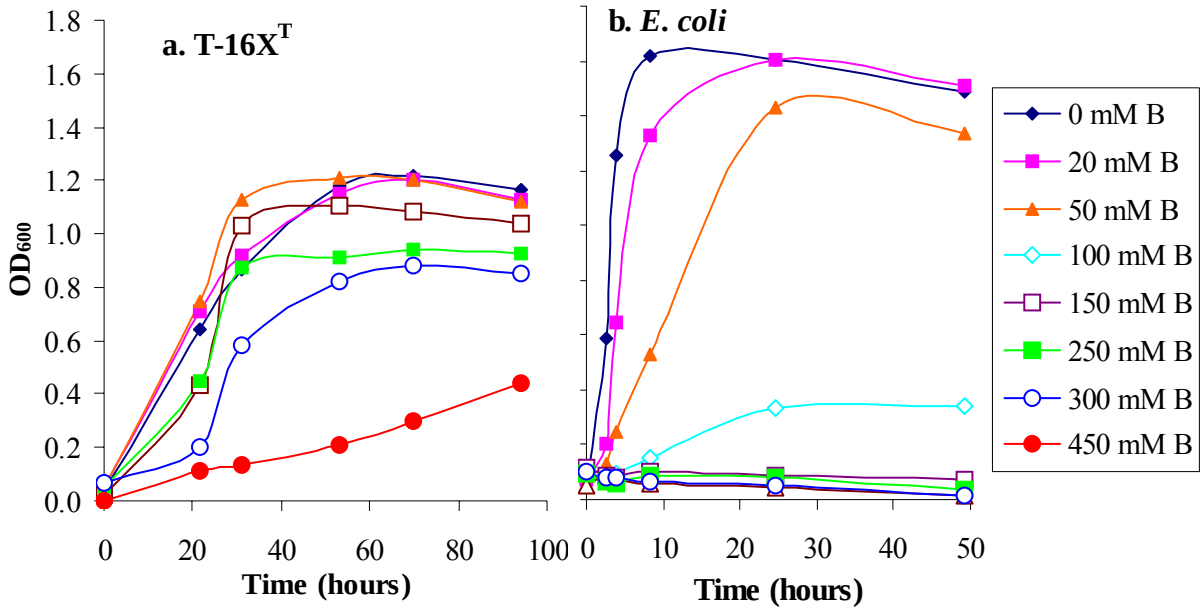

Figure 4.2

Growth curves for (a) strain T-16X^T and (b) *Escherichia coli* control grown at different levels of boron supply (mM) in tryptic soy broth (TSB) medium, (pH 7.4 ± 0.1) at 30ºC and with vigorous shaking. For simplicity, the overlapping lines of growth at 100 and 200 mM boron in (a) are deleted, and lines at >150 mM boron in (b) are overlapping.

(Ahmed *et al*., 2007a), *Bacillus boroniphilus* is 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 (Stainer *et al*., 1966); however, the biological functions requiring boron in plants and microbes are not clear, despite the fact that boron is required for growth in some organisms.

Physiological characteristics

The ranges of pH, NaCl concentration, and temperature for the growth of strain T-16X^T were determined as described previously (Ahmed *et al*., 2007a). Motility, catalase and oxidase tests, resistance to antibiotics, and enzyme activity were also evaluated following previously described procedures (Ahmed *et al*., 2007a). 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 4.1) and highly boron-tolerant (Figure 4.2). The strain withstood 11% (w/v) NaCl and grew optimally in the presence of 1 to 3% NaCl. Because it exhibited optimum growth at alkaline pH (range 7.5–8.5) and was able to grow at pH greater than 9.0, it was characterized as alkali-tolerant, 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 (Difco), tryptic soy agar (TSA), and nutrient agar (NA), although the growth was slow and colony size was small. Strain T-16X^T shares many characteristics with members of the genus *Gracilibacillus*. The characteristics that differentiated strain T-16X^T from its closely related type strains are shown in Table 4.1. Additional characteristics are included in the

description of *G. boraciitolerans* sp. nov. In addition to its boron tolerance, the major characteristic differentiating strain T-16X^T from its closely related type strains was a positive Voges–Proskauer test.

Table 4.1. Characteristics that differentiate *Gracilibacillus boraciitolerans* sp. nov. and its most closely related, previously described species.

1, *Gracilibacillus boraciitolerans* sp. nov. T-16X^T; **2,** *G. orientalis* CCM 7326^T (data from Carrasco *et al*., 2006); **3,** *G. dipsosauri* DSM 11125^T (data from Deutch, 1994 and Lawson *et al*., 1996); **4,** *G. halotolerans* DSM 11805^T (data from Wainø *et al*., 1999). S, spherical; E, ellipsoidal; T, terminal position of endospore; +, positive; –, negative; nd, no data.

Characteristic	1	2	3	4
Size: length (filaments) × 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 and position	S/E, T	S, T	S, T	E, T
Growth at:				
NaCl range (%) (optimum)	0–11 (0.5–3.0)	1–20 (10)	0–15	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)	(7.5)	5–10 (7.5)
Oxidase activity	+	–	+	+
Hydrolysis of:				
Gelatin	–	+	+	+
Urea	–	–	–	+
Production of H_2S	–	–	–	+
Nitrate reduction	–	–	+	+
Voges-Proskauer test	+	nd	–	–
G+C content (mol%)	35.8	37.1	39.4	38

† Results of this study.

16S rRNA sequencing and phylogenetic analysis

A nearly complete 16S rRNA gene fragment (1500 nucleotides) of strain T-16X^T was amplified by polymerase chain reaction (PCR) according to the protocol described by Katsivela *et al.* (1999). The purified PCR product was sequenced as previously described (Ahmed *et al.*, 2007a). 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 CLUSTALX (1.8 msw) software (Thompson *et al*., 1997) and editing using the BioEdit (Hall, 1999) software package to exclude ambiguous positions and gaps during calculations. Ultimately, 1302 bp of 1500 bp total were used to calculate evolutionary distances and K_{nuc} values (Kimura, 1980) and to construct a phylogenetic tree using the neighbor-joining method (Saitou & Nei, 1987) in the PHYLIP software package (Felsenstein, 2005). The tree was plotted using NJ Plot software. To assess the stability of the phylogenetic tree, a bootstrap analysis (Felsenstein, 2005) was performed using 1000 resamplings of the neighbor-joining data. The topologies of the phylogenetic trees were also generated using maximum-likelihood and maximum-parsimony algorithms, which generated relationships similar to those constructed using the neighbor-joining method for the novel strain (Figures 4.3a & 4.3b). 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* (AM040716), followed by 95.5% with *G. halotolerans* (AF036922), 95.4% with *G. dipsosauri* (X82436), and 95.7% with *Paraliobacillus ryukyuensis* (AB087828), after alignment. 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 clade with *G. orientalis* (AM040716) at a 64% bootstrap value (Figure 4.3). The phylogenetic position of strain T-16X^T with *G. orientalis* was also supported by cellular fatty acid profiles (Table 4.2), in addition to other physiological and chemotaxonomic data.

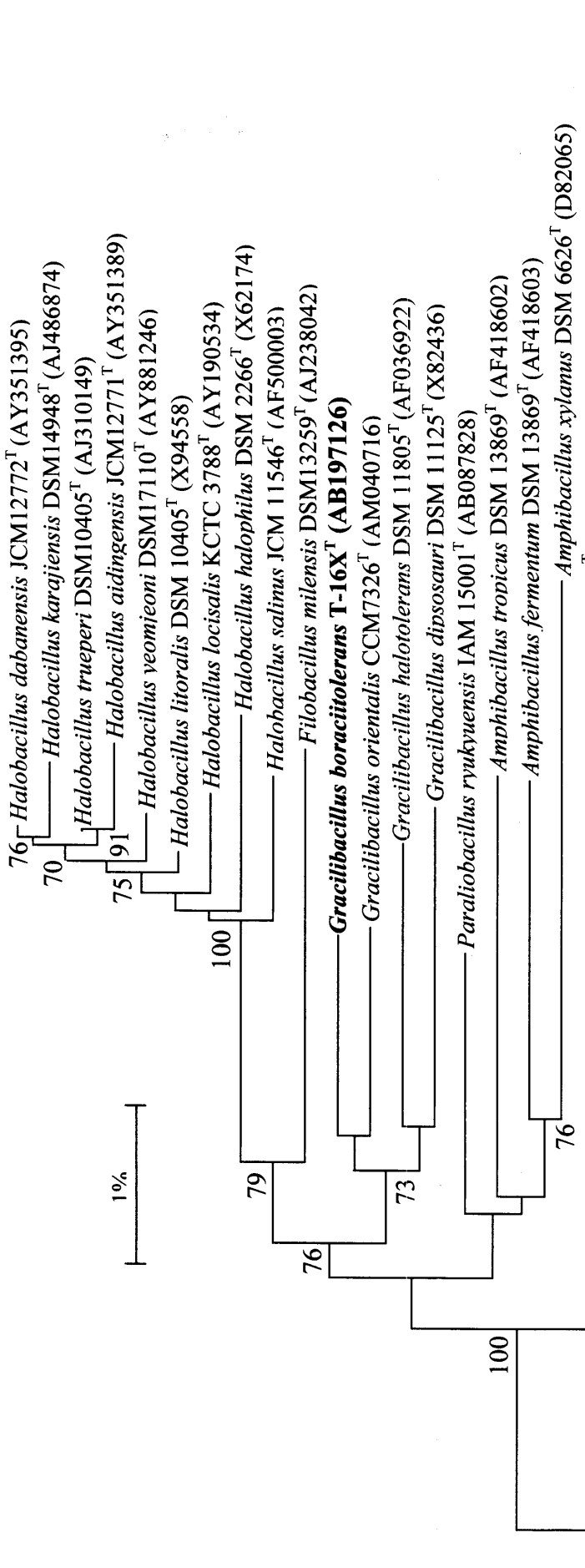

Figure 4.3. Phylogenetic tree showing the interrelationships of *Gracilibacillus boraciitolerans* sp. nov. and its closely related previously described species inferred from 16S rRNA gene sequences. The tree was constructed using the **neighbor-joining method** contained in the PHYLIP software package (Felsenstein, 2005), based on a comparison of approximately 1302 bp, and plotted using NJ Plot. Bootstrap values greater than 70%, expressed as a percentage of 1000 replications, are given at the branching points. *Geobacillus pallidus* (Z26930) is the outgroup. Bar, 1% sequence divergence.

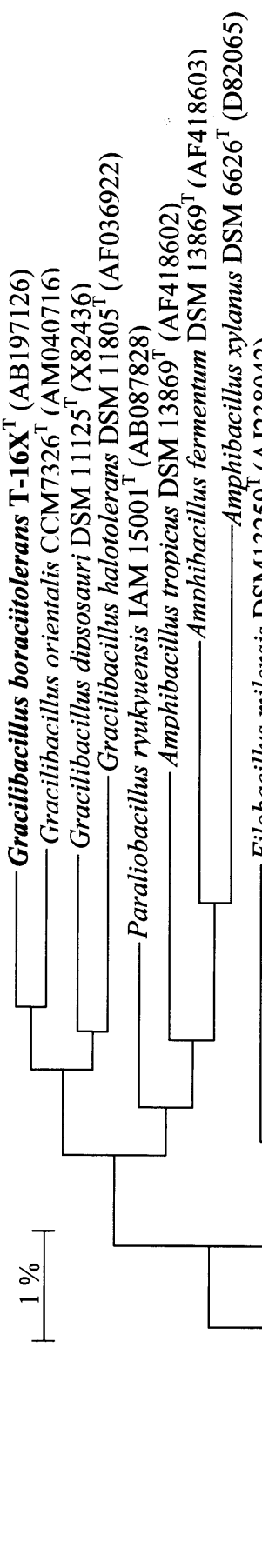

Figure 4.3a. Phylogenetic tree showing the interrelationships of *Gracilibacillus boraciitolerans* sp. nov. and its closely related species inferred from 16S rRNA gene sequences. The tree was constructed using the **maximum-likelihood method** contained in the PHYLIP software package (Felsenstein, 2005) based on a comparison of approximately 1302 bp, and plotted using NJ Plot. *Geobacillus pallidus* (Z26930) is the outgroup. Bar, 1% sequence divergence.

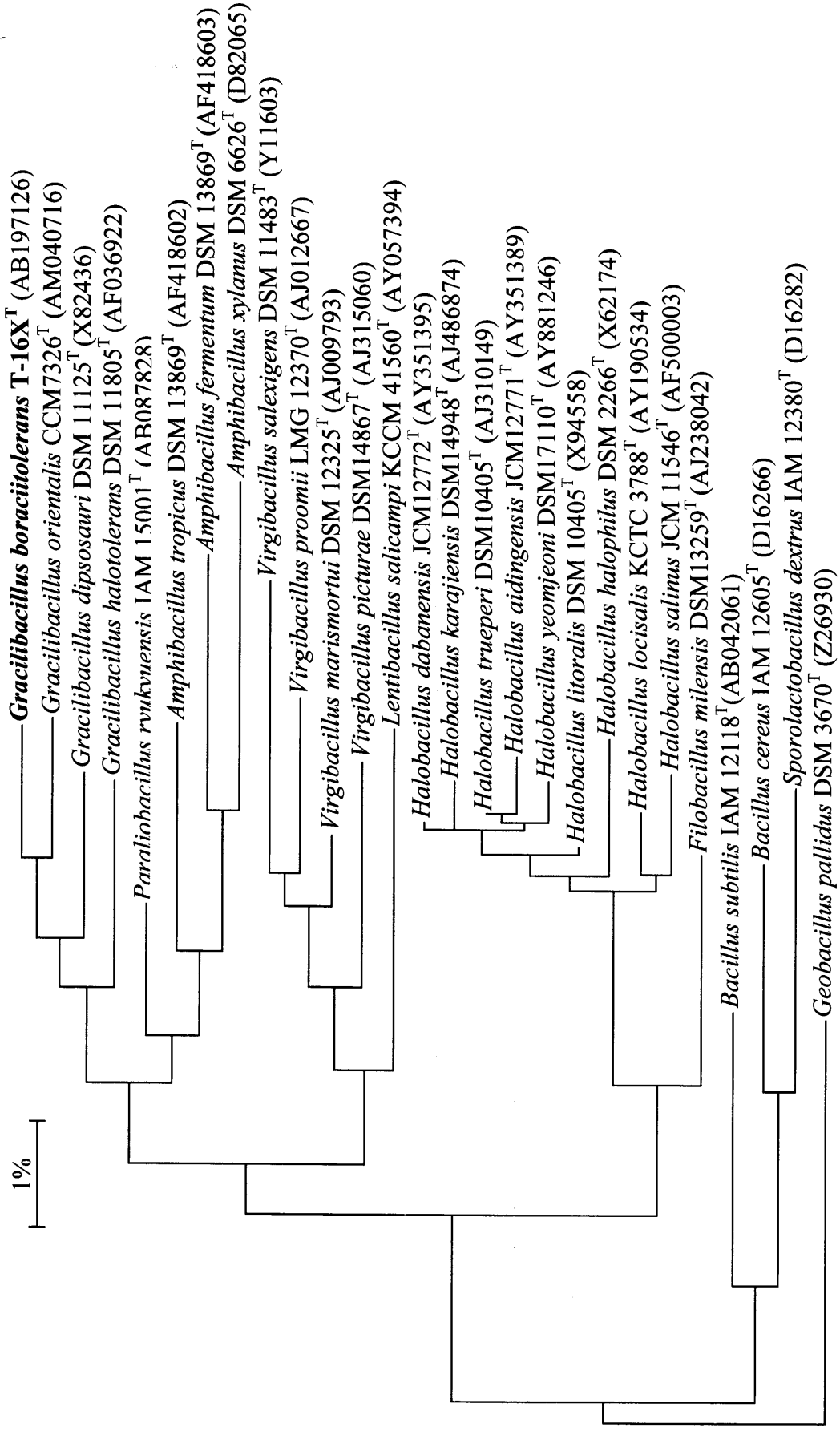

Figure 4.3b. Phylogenetic tree showing the interrelationships of *Gracilibacillus boraciitolerans* sp. nov. and its closely related species inferred from 16S rRNA gene sequences. The tree was constructed using the **parsimony method** contained in the PHYLIP software package (Felsenstein, 2005) based on a comparison of approximately 1302 bp, and plotted using NJ Plot. *Geobacillus pallidus* (Z26930) is the outgroup. Bar, 1% sequence divergence.

Chemotaxonomic analyses

A whole-cell fatty acid profiles for strain T-16X^T and *Gracilibacillus orientalis* CCM 7326^T were analyzed from freeze-dried cell material grown in MB or MB+1.5% NaCl for 24 h using the GC-based Microbial Identification system (MIDI) according to the manufacturer's instructions. Strain T-16X^T was composed predominantly of branched-chain fatty acids

Table 4.2. Cellular fatty acid composition (% of total fatty acids)† of strainT-16X^T and its most closely related species.

Characteristic	T-16X^T		*G. orientalis*	*G. dipsosauri*‡	*G. halotolerans*‡
	MB	MB + 1.5%	MB + 1.5%	MB	MB
Straight-chain fatty acids:					
$C_{14:0}$	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 fatty acids:					
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 fatty acids:					
$C_{16:1}$ ω 7*c* alcohol	nd	0.1	0.8	nd	nd
$C_{16:1}$ ω 11*c*	0.2	0.3	2.3	tr*	3.0*
$C_{18:1}$ ω 9 *c*	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

†Only fatty acids comprising >0.50% of the total in at least one strain are included.
‡Data from Wainø *et al.* (1999).
* This component was reported only as $C_{16:1}$ by Wainø *et al.* (1999).
** This component was reported only as "Isomers of $C_{18:1}$" by Wainø *et al.* (1999) indicating 'Sum of two different isomers, which are resolved as two components (2.73 and 2.45%)'.
¶Iso-$C_{15:0}$ 2-OH/$C_{16:1}$ ω7*c*.
§Iso-$C_{17:0}$ I/anteiso-$C_{17:0}$ B.
tr, trace amount; nd, not detected.

(Table 4.2), 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. However, it differed from other members of *Gracilibacillus* in terms of morphological, physiological, and chemotaxonomic features (Table 4.1). The fatty acid profile was quite similar to that of other members of *Gracilibacillus*, especially *G. orientalis*, in that it contained the same components, but at slightly different amounts (Table 4.2). Its phylogenetic position (Figure 4.3) was also closely associated with *G. orientalis*. *Halobacillus* species possess the unsaturated fatty acids $C_{16:1}\omega7c$ alcohol, and $C_{17:1}\omega10c$, which were absent from the profile of strain T-16X^T and other members of *Gracilibacillus*.

MK-7 (90%) was determined as the predominant respiratory quinone system in the novel strain using the method described by Xie and Yokota (2003), although MK-6 (10%) was also detected as a minor component. Cell wall peptidoglycans were analyzed for amino acid content using two-dimensional thin-layer chromatography (2-D TLC) and high-performance liquid chromatography (HPLC; Shimadzu) as described elsewhere (Schleifer & Kandler, 1972; Groth *et al*., 1996). The peptidoglycans of strain T-16X^T contained glutamic acid, *m*-diaminopimelic acid, alanine, and muramic acid (Figure 4.4) in the molar

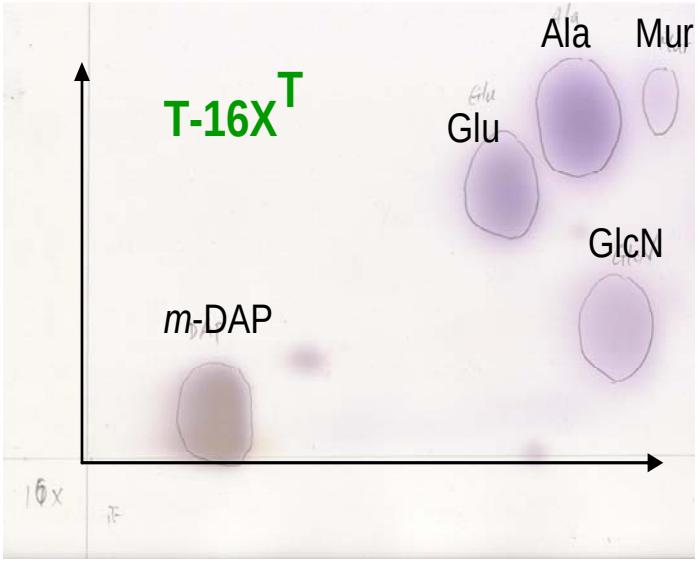

Figure 4.4.

Cell wall peptidoglycans amino acids of the strain T-16X^T as analyzed by using two-dimensional thin-layer chromatography (2-D TLC).

ratio of 1.0:1.1:2.0:0.4, respectively, as the diagnostic amino acids representing peptidoglycan type A1γ (i.e., directly cross-linked *meso*-diaminopimelic acid; Schleifer & Kandler, 1972).

The polar lipids were extracted and purified from 100 mg of dried cells by the procedure of Minnikin *et al.* (1984) and examined by 2-D TLC, using Kieselgel 60 F254 plates (E. Merck, Darmstadt, Germany), as described by Kudo (2001). The polar lipid profile of strain T-16X^T predominantly consisted of diphosphatidylglycerol and phosphatidylglycerol (Figure 4.5). Additionally, an unknown aminolipid (AL1) and three unknown polar lipids (L1, L2, L3) were detected, which could not be detected with any of the specific spray reagents employed. However, no reagent for detection of glycolipids was used. The genus type species *G. halotolerans* was also diagnosed with MK-7 as the predominant respiratory quinone system, *m*-diaminopimelic acid in cell wall peptidoglycans of type A1γ, and the major polar lipids of 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* but lack of phosphatidylethanolamine, an

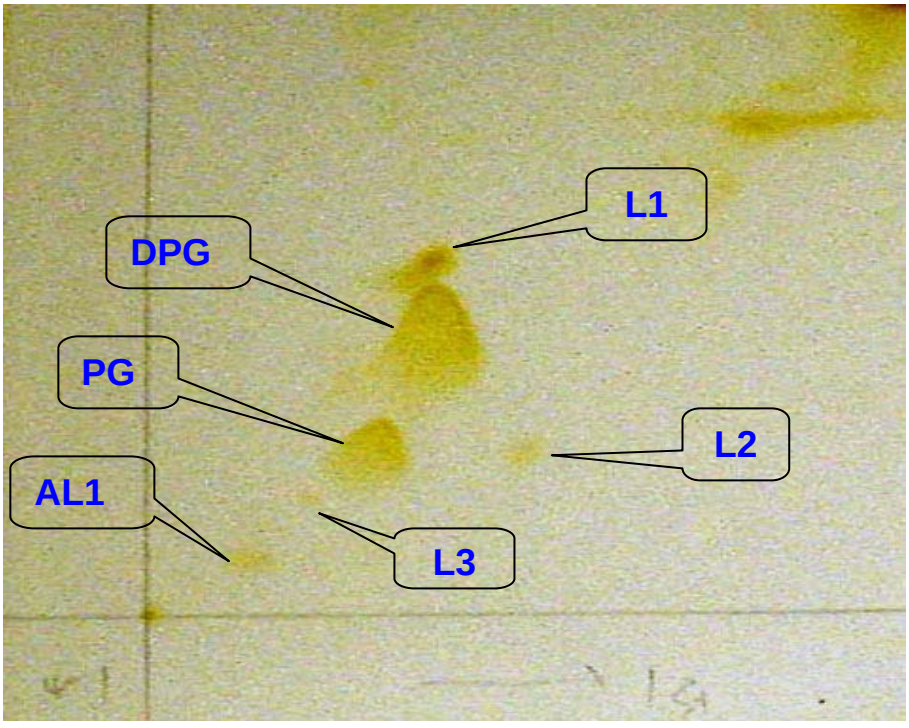

Figure 4.5

Polar lipid profile of the strain T-16X^T analyzed using two-dimensional thin layer chromatography. DPG, diphosphatidyl glycerol; PG, phophatidyl glycerol; L1–L3, unknown polar lipids; AL1, unknown aminolipid.

unknown phospholipids and two unknown aminophospholipids reported to be present in *G. orientalis* (Carrasco *et al.,* 2006) distinguished strain T-16X^T from its phylogenetic neighbour (Figure 4.3). These characteristics also fit to many other bacilli. Thus, they do not indicate that the strain is a member of *Gracilibacillus.*

G+C content and DNA-DNA hybridization

The G+C content of strain T-16X^T was 35.8 mol% as determined using a previously described procedure (Ahmed *et al*., 2007a). The other three species of the genus *Gracilibacillus* have G+C contents in the range of 37 to 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* (Figure 4.3), and critical analysis of other chemotaxonomic data (Table 4.1) 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 species was isolated using QIAGEN Genomic-tip 500/G following the manufacturer's protocol, with a minor modification in which RNase T_1 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-labeled 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 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 is a novel species (Stackebrandt & Goebel, 1994). The morphological, chemotaxonomical, phylogenetic, and genotypic features of strain T16X^T suggest that it belongs to the genus *Gracilibacillus*; we propose the name *Gracilibacillus boraciitolerans* sp. nov. for the type strain T-16X^T described hereafter.

Description of *Gracilibacillus boraciitolerans* sp. nov.

Gracilibacillus boraciitolerans (bo.ra.ci'ito.le.rans. N.L. n. *boracium*, boron; L. part. adj. *tolerans* tolerating; N.L. part. Adj. *boraciitolerans*, boron-tolerating)

Cells are motile using a long monotrichous flagellum; Gram-positive, short rods, 2.0–4.5 μm in length, 0.3–0.9 μm in diameter, occurring singly, occasionally in pairs; filamentous cells also occur. Spherical endospores are produced in a non-swollen or slightly swollen sporangium at 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 of growth on BUG agar medium (pH 7.5) at 30ºC. 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. The strain grows optimally at 25–28ºC; the temperature range for growth is 16–37ºC; no growth occurs at ≥45ºC and little growth at 16ºC after several days. The optimum pH for growth is 7.5–8.5, with a range of 6.0 to 10.0. The strain can tolerate 0–450 mM boron, but grows optimally in the absence of boron. The NaCl salt-tolerance range is 0–11% (w/v), indicating that the strain is moderately halotolerant; it can grow on Marine agar 2216 (Difco), TSA, and NA (with or without boron or NaCl salt). Oxidase, catalase, Voges–Proskauer, and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) tests are positive, whereas tests for production of indole and H_2S, NO_3 reduction, lysine and ornithine decarboxylases, arginine dehydrolase, tryptophan deaminase, citrate utilization, hydrolysis of gelatin, and urea are negative (API 20E). The strain can produce acid from L-arabinose, D-ribose, glucose, D-mannose, esculin, D-cellobiose, D-maltose, D-lactose, D-melibiose, and D-trehalose, whereas it is weakly positive for acid production from D-xylose, methyl-β-D-xylopyranoside, D-frutose, D-mannitol, and D-sorbitol (API 50CHB). The strain 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, α- and β-methyl D-galactoside, α- and β-methyl D-glucoside, D,L-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%; $C_{18:1}$ ω9*c*, 1.0% and traces (less than 1%) of some other types. The cell wall contains peptidoglycan with *m*-diaminopimelic acid as the diagnostic amino acid representing the cell wall peptidoglycan type A1γ. 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 as well. 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 Strip). The strain is resistant to penicillin, amoxicillin, and metronidazol (ATB-VET Strip). The G+C content of the strain is 35.8 mol%, as determined using HPLC.

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

Acknowledgments

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CHAPTER V

Chimaereicella boritolerans sp. nov., a novel boron-tolerant and alkaliphilic bacterium of the family Flavobacteriaceae, isolated from soil

ABSTRACT

A non-motile, Gram-negative, boron (B)-tolerant and alkaliphilic bacterium was isolated from soil of the Hisarcik area in the Kutahya Province of Turkey that was naturally high in B minerals. The novel isolate, designated T-22^{T*}, formed rod-shaped cells, was catalase and oxidase positive, and tolerated up to 300 mM B. The strain also survived on agar medium containing up to 3% (w/v) NaCl salt. The pH range for the growth of this strain was 6.5–10.0 (optimum pH 8.0–9.0), and the temperature range was 16–37ºC (optimum 28–30ºC). A phylogenetic analysis based on 16S rRNA gene sequences revealed clear affiliation with the genus *Chimaereicella*, with 97.3% sequence similarity, which was the highest similarity among the cultivated bacteria. The DNA–DNA homology with *C. alkaliphila* was 28.3%. The major respiratory quinone system was MK-7, and the predominant cellular fatty acids were iso-$C_{15:0}$, iso-$C_{17:1}$ ω9*c*, iso-$C_{17:0}$ 3-OH, and iso-$C_{15:0}$ 2-OH and/or iso-$C_{16:1}$ ω7*c*. The DNA G+C content was 42.5 mol%. Based on the phylogenetic analysis and physiological, chemotaxonomic, and genetic data, we concluded that the novel strain T-22^T (DSM 17298^T = NBRC 101277^T = ATCC BAA-1189^T) should be classified in the genus *Chimaereicella*, and we propose the name *Chimaereicella boritolerans* sp. nov. for this new species.

Abbreviations

B: boron, MA: marine agar 2216 (Difco), MB: marine broth 2216 (Difco), nts: nucleotides, SEM: scanning electron microscopy.

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* The DDBJ/EMBL/GenBank accession number for the 16S rRNA gene sequence of the strain T-22^T (NBRC 101277^T = DSM 17298^T = ATCC BAA-1189^T) is AB197852.

INTRODUCTION

Several classes of microorganisms have been recognized as extremophiles, which inhabit environments with extreme levels of salinity, pH, pressure, heat, cold, and even radiation. These extremophiles provide a novel source of discoveries in the basic and applied sciences. We present the boron (B) tolerance of bacteria as a new frontier in extremophiles. The toxic effects of high concentrations of boric acid on living cells are well established (Nable *et al*., 1997). The biological functions requiring B in microbes and plants are not clear, despite the fact that B is required by some organisms as an essential nutrient (Warington, 1923; Rowe & Eckhert, 1999). Although B salts have often been used in microbial growth media (Stanier *et al*., 1966), the B essentiality and tolerance of B toxicity in bacteria has only been reported recently (Ahmed *et al.,* 2007a & b).

Recently, the genus *Chimaereicella* was described in the family Flavobacteriaceae; only one of its species, which was isolated from a non-saline but highly alkaline aquatic environment, is currently characterized (Tiago *et al.,* 2006). Most members of this cluster, also known as the *Cytophaga–Flexibacter–Bacteroides* (CFB) group (Garrity & Holt, 2001), are Gram-negative and originated from marine and marine-derived surface waters (Bowman & Nichols, 2005). These organisms play a major role in the remineralization of organic matter (Kirchman, 2002).

In the course of exploring the bacterial diversity for B tolerance, we isolated a novel species of Gram-negative, alkaliphilic, and highly B-tolerant bacterium from soil sampled from the Hisarcik area in the Kutahya Province of Turkey. This area is naturally high in B minerals (Çöl & Çöl, 2003). Based upon the data obtained, we propose that the strain occurs within the genus *Chimaereicella* and classified it as *Chimaereicella boritolerans* sp. nov.

Isolation and morphology

Strain T-22^T was isolated from soil according to previously described isolation and enrichment procedures (Ahmed *et al*., 2007a). After isolation, the strain was cultured

routinely on R3V-A medium (see composition in Tiago *et al.*, 2006) or marine agar 2216 (MA, Difco) medium (pH 8.0) at 30ºC, or stored in glycerol stock (35%, w/v) at –80ºC.

The motility and morphology of cells were observed using phase-contrast microscopy and scanning electron microscopy (SEM). Gram staining was performed according to Hucker's modified method (Cowan, 1974). Colonial morphology was observed on isolated colonies grown on R3V-A or MA medium (pH 8.0). For SEM observation, cells grown on agar plates for 5, 10, and 22 days were removed, washed with phosphate-buffered saline (PBS) solution, and fixed with OsO_4. After dehydration by sequential washing with ethanol (50–100%) that was exchanged to 100% isoamyl acetate, the sample was treated with platinum before observation using SEM (JEOL model JSM-6700F, Tokyo, Japan). The

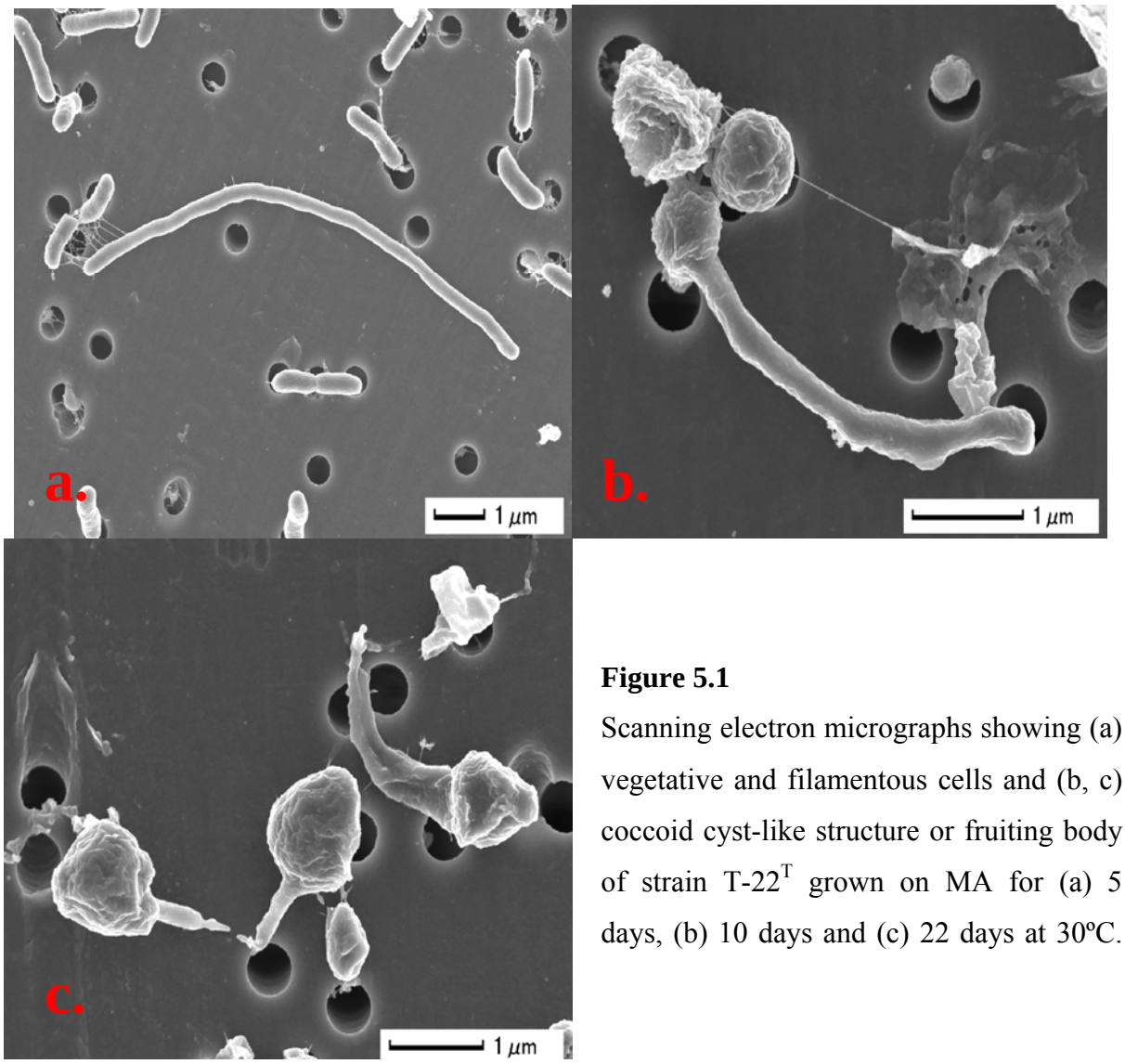

Figure 5.1

Scanning electron micrographs showing (a) vegetative and filamentous cells and (b, c) coccoid cyst-like structure or fruiting body of strain T-22^T grown on MA for (a) 5 days, (b) 10 days and (c) 22 days at 30ºC.

young cells of strain T-22^T at mid-logarithmic phase were non-motile and rod shaped, with a length of 1.2–3.4 μm and width of 0.3–0.4 μm; however, long filamentous cells (up to 15 μm) were also observed (Figure 5.1a). The older cells (22 days) produced coccoid cyst-like structures or fruiting-body-like structures (Figure 5.1b & c), with a diameter range of 0.76–0.90 μm. The colonies produced red pigmentation that was diffusible in the medium.

Phenotypic features

The pH range for growth of the strain was determined on RV3-A or MA media with pH ranging from 4.0 to 10.0 (in pH increments of 0.5), adjusted as described by Tiago *et al*. (2006) at 30ºC. The temperature range for growth of the strain was determined on agar medium with pH 8.0 by incubation at various temperatures, i.e., 4, 10, 16, 20, 25, 28, 30, 32, 37, 45, and 50°C. The growth of strain T-22^T was also examined at levels of NaCl ranging from 0 to 7% (w/v, in 1% increments) on RV3-A medium at pH 8.0 and 30ºC. Strain T-22^T grew on agar with a pH range of 6.5 to 10.0; however, it exhibited optimum growth at an alkaline pH range of 8.0–9.0, so it was characterized as alkaliphilic according to the definition by Jones *et al.* (1994). The intensity of red pigmentation decreased at pH 10.0, whereas it was maximal at neutral pH. The optimum temperature for growth was 28–30ºC. Slight growth was observed at 16ºC or 37ºC after several days; however, no growth was observed at temperatures of 10ºC or 40ºC. Strain T-22^T tolerated up to 3% NaCl.

Physiological characteristics

Catalase and oxidase tests were performed following previously described procedures (Ahmed *et al*., 2007a). The strain was positive for catalase and cytochrome oxidase tests. Carbon source use was investigated using API 50 CH strips (bioMérieux, France), using 0.1 M Tris (hydroxymethyl) aminomethane–Tris buffer (pH 8.0) supplemented with 0.3% (w/v) agar, 0.7% yeast nitrogen base, and 0.05% yeast extract as a minimal medium. Acid production on various substrates was determined using 50CHB/E medium with or without adding 0.02 M Tris (hydroxymethyl) aminomethane–Tris buffer (pH 8.0) following the manufacturer's instructions and as described by Tiago *et al.* (2006).

Results were recorded after 24 h, 48 h, and 5 days of incubation at 30ºC. Other physiological tests were performed using the API 20NE (bioMérieux) system using the minimal medium as described by Tiago *et al.* (2006). Resistance to antibiotics and enzyme activity were evaluated using ATB-VET and API-ZYM strips (bioMérieux), respectively, 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. Strain T-22^T showed many common and differentiating characteristics with the closely related species *Chimaereicella alkaliphila* (Table 5.1); additional features are included in the description of *Chimaereicella boritolerans* sp. nov.

Table 5.1. Physiological characteristics of two species in the genus *Chimaereicella*.

Both species are positive for: catalase, oxidase, esculin, β-galactosidase; both species produce acid from: D-galactose, D-glucose, D-cellobiose, D-maltose, D-lactose, potassium 5-ketogluconate, gentibiose, D-turanose; both species are resistant to the antibiotic kanamicin. Both species are negative for: urease, indole from L-tryptophane; both species do not produce acid from: glycerol, erythritol, D- and L-arabinose, D-ribose, D- and L-xylose, D-adonitol, L-sorbose, dulcitol, inositol, D-mannitol, D-sorbitol, amygdalin, D-melezitose, xylitol, D-tagatose, D- and L-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate, methyl-β-D-xylopyranoside; both species cannot use: glycerol, erythritol, D-arabinose, D-ribose, D- and L-xylose, methyl-β-D-xylopyranoside, D-galactose, D-glucose, L-sorbose, L-rhamnose, inositol, *N*-acetylglucosamine, D-lactose, D-melibiose, D-trehalose, D-rafinose, D-turanose, potassium gluconate, L-fucose, D-tagatose; both species are sensitive to the antibiotics: amoxycilin, colistin, doxycyclin, ofloxacin, streptomycin, tetracyclin. +, Positive result; –, Negative result; (+), weakly positive.

Characteristic	***Chimaereicella boritolerans* sp. nov.**	***Chimaereicella alkaliphila****
Cell size; length (filamentous) × width, µm	1.2–3.4 (15) × 0.3–0.4	2.1–3.3 × 0.5
Growth at:		
Boron conc. (mM B)	300	50 †
pH range (optimum)	6.5–10 (8–9)	7.5–11 (8)
Hydrolysis of gelatin	–	+
Nitrate reduction	–	+
Acid from:		
D-fructose	–	+
D-mannose	(+)	–
L-rhamnose	(+)	–
Methyl-αD-mannopyranoside	(+)	–
N-acetylglucosamine	(+)	–
Esculin	+	–

Salicin	–	+
D-melibiose	–	+
D-saccharose (sucrose)	–	+
D-trehalose	–	+
Inulin	+	–
D-rafinose	+	–
Amidon (starch)	+	–
Glycogen	(+)	–
D-lyxose	(+)	–
Use of:		
L-arabinose	–	+
D-adonitol	–	+
D-fructose	–	(+)
D-mannose	–	(+)
Dulcitol	(+)	–
Inositol	–	–
D-mannitol	+	–
D-sorbitol	–	+
Methyl-αD-glucopyranoside	–	+
Amygdalin	+	–
Arbutin	+	–
Esculin	+	–
D-cellobiose	–	+
D-maltose	(+)	–
Inulin	+	–
D-melezitose	+	–
Amidon (starch)	(+)	–
Glycogen	(+)	–
Xylitol	(+)	–
Gentibiose	–	+
D-lyxose	(+)	–
D-fucose	(+)	–
L-arabitol	–	+
Potassium 2-ketogluconate	–	+
Potassium 5-ketogluconate	–	+
Resistance to (μg ml^{-1}):		
Cephalothin (8)	–	+
Chloramphenicol (30)	–	+
Gentamicin (4)	(+)	–
Penicillin (0.25)	(+)	–
DNA G+C content (mol%)	42.5	43.5

* Data from Tiago *et al.* (2006).

† Data of these studies.

16S rRNA sequencing and phylogenetic analysis

An almost complete 16S rRNA gene fragment (1489 nucleotides, nts) of strain T-22^T was amplified by polymerase chain reaction (PCR) and sequenced as described by Ahmed *et al.* (2007a) using an ABI PRISM® 3730XL Genetic Analyzer (Applied Biosystems). The consensus sequence obtained using the DNASIS Pro software package (Hitachi Software Engineering, Japan) was compared with sequences of closely related type strains retrieved from the DDBJ/EMBL database using BLAST searches. The multiple-sequence alignment was produced by CLUSTAL X (1.8 msw) software (Thompson *et al.*, 1997). Ambiguous positions and gaps were discarded and sequence identities were calculated using BioEdit software (Hall, 1999). The evolutionary distances and K_{nuc} values (Kimura, 1980) were generated from unambiguous alignable data of 1342 nts, and phylogenetic trees were constructed using the neighbor-joining method (Saitou & Nei, 1987) and maximum parsimony and maximum likelihood algorithms (Felsenstein, 2005). To assess the stability of relationships, a bootstrap analysis (confidence values estimated from 1000 replications of each sequence) was performed using PHYLIP software (Felsenstein, 2005) for the tree topology of the neighbor-joining data. The phylogenetic trees were plotted using the NJ Plot software.

Comparison of the 16S rRNA sequence data with those of closely related type species showed that strain T-22^T belonged to the genus *Chimaereicella*, with the highest similarity in the 16S rRNA sequence of 97.4% with that of *C. alkaliphila* LMG 22794^T (AJ717393). Sequence similarity calculations indicated that strain T-22^T was equidistant from species of the genus *Algoriphagus* (similarity range of 94.0–95.4%) and the genus *Hongiella* (similarity range of 93.6–94.6%) and occupied the phylogenetic position of *C. alkaliphila* LMG 22794^T, achieving a 100% bootstrap value indicating high reliability of the tree topology (Figure 5.2). The topologies of the phylogenetic trees generated using the maximum likelihood and maximum parsimony algorithms were similar to that constructed using the neighbor-joining method (Figures 5.2a & 5.2b), especially for the clade comprised of species of *Chimaereicella*. These analyses strongly support the affiliation of strain T-22^T with the genus *Chimaereicella.*

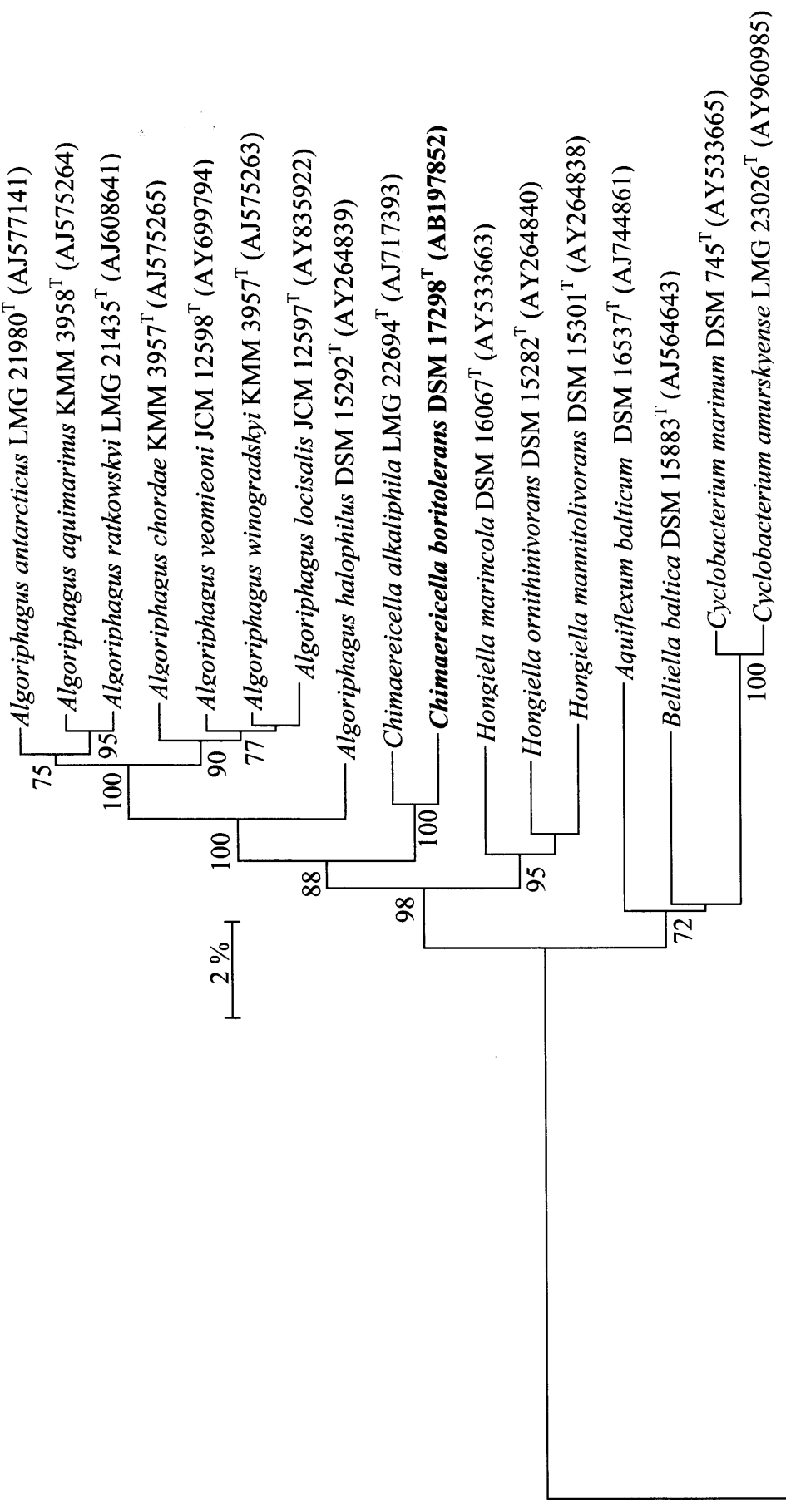

Figure 5.2. Phylogenetic tree showing the inter-relationships of *Chimaereicella boritolerans* sp. nov. and its closest relatives inferred from 16S rRNA gene sequences. Data with gaps were removed during alignment using BioEdit software, and the tree was constructed using the **neighbor-joining method** with PHYLIP software (Felsenstein, 2005) based on a comparison of approximately 1342 nucleotides and plotted with NJ Plot. Bootstrap values >70%, expressed as a percentage of 1000 replications, are given at the branching points. The sequence of *Escherichia coli* (J01859) is used as the outgroup. Bar, 2% sequence divergence.

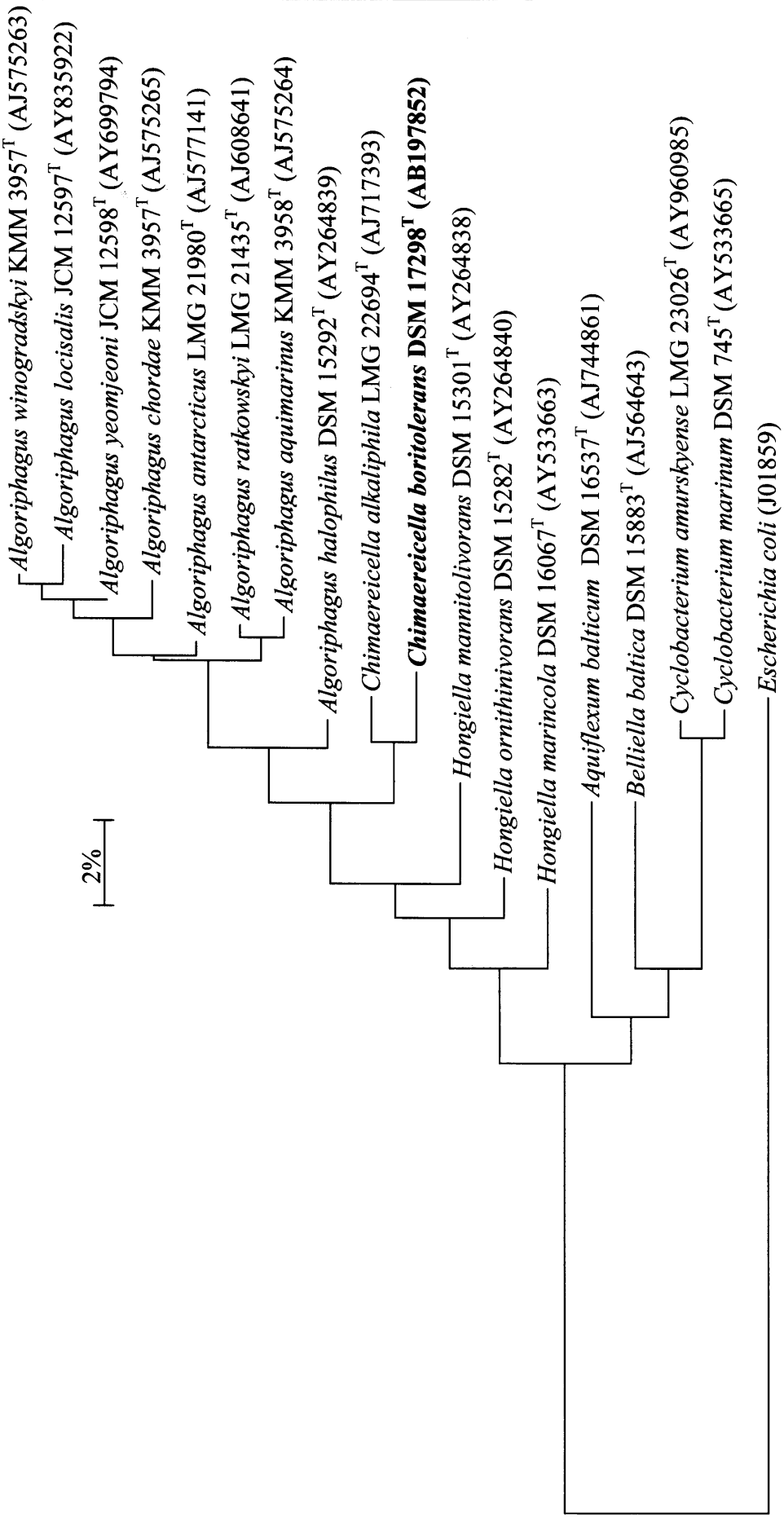

Figure 5.2a. Phylogenetic tree showing the inter-relationships of *Chimaereicella boritolerans* sp. nov. and its closest relatives inferred from 16S rRNA gene sequences. Data with gaps were removed during alignment using BioEdit software, and the tree was constructed using the **maximum likelihood algorithm** with PHYLIP software (Felsenstein, 2005) based on a comparison of approximately 1342 nucleotides and plotted with NJ Plot. The sequence of *Escherichia coli* (J01859) is used as the outgroup. Bar, 2% sequence divergence.

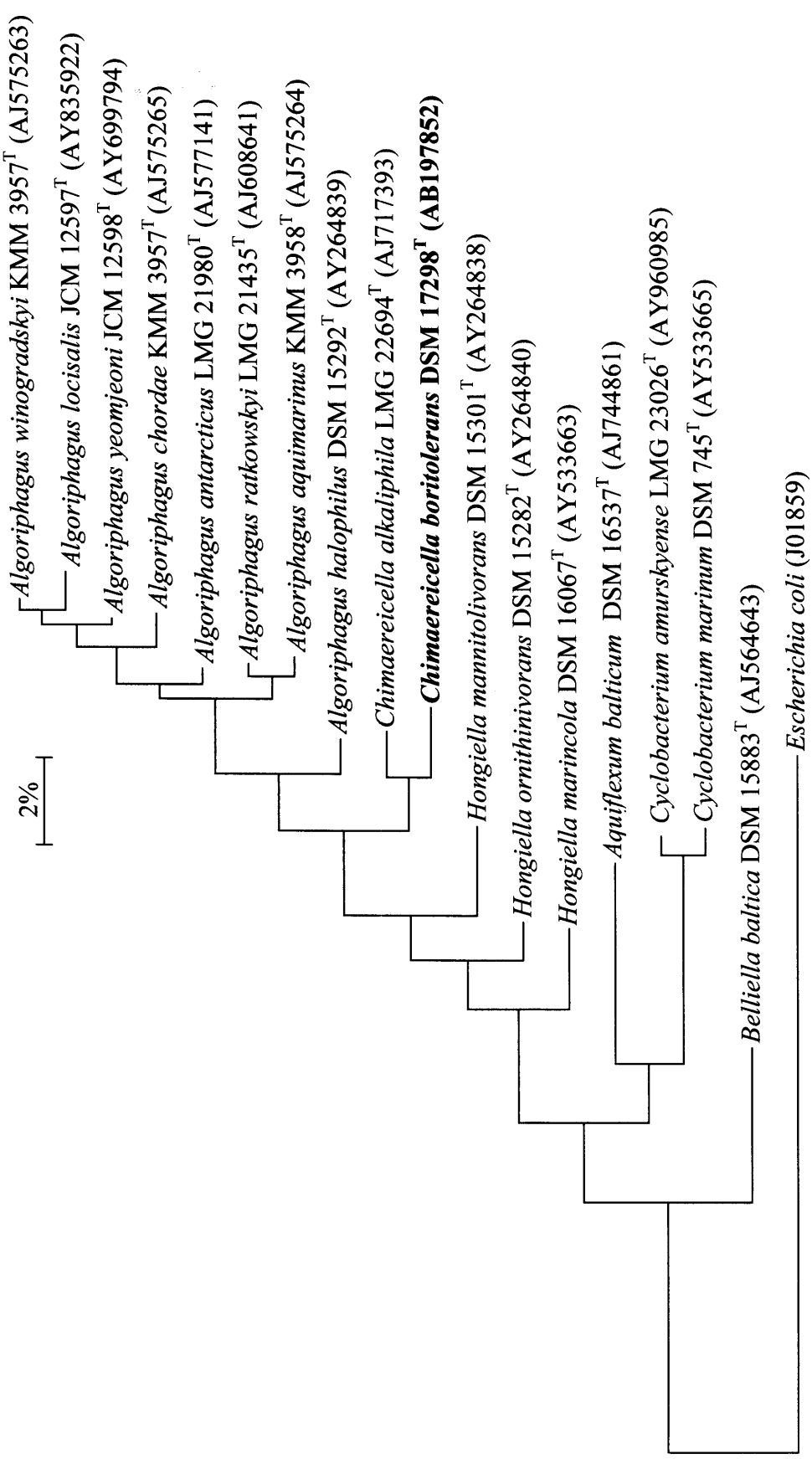

Figure 5.2b. Phylogenetic tree showing the inter-relationships of *Chimaereicella boritolerans* sp. nov. and its closest relatives inferred from 16S rRNA gene sequences. Data with gaps were removed during alignment using BioEdit software, and the tree was constructed using the **parsimony algorithm** with PHYLIP software (Felsenstein, 2005) based on a comparison of approximately 1342 nucleotides and plotted with NJ Plot. The sequence of *Escherichia coli* (J01859) is used as the outgroup. Bar, 2% sequence divergence.

Chemotaxonomic analyses

The major respiratory quinone system in strain T-22^T was MK-7, as determined using the protocol described by Xie and Yokota (2003). To analyze the fatty acid profile, methyl ester derivatives were prepared by saponification, methylation, and extraction from 10 mg of freeze-dried cell material harvested from either R3V-A or marine broth (MB, Difco) medium. The fatty acid methyl esters were analyzed using the gas chromatography-based Microbial Identification system (MIDI) following the manufacturer's instructions. The predominant cellular fatty acids detected in strain T-22^T were branched chains, with the major entities iso-$C_{15:0}$, iso-$C_{17:0}$ ω9*c*, iso-$C_{15:0}$ 2-OH or $C_{16:0}$ ω7*c*, iso-$C_{17:0}$ 3-OH, and iso-$C_{16:0}$ (Table 5.2).

Table 5.2. Cellular fatty acid composition (percentage of total) of *Chimaereicella boritolerans* sp. nov. T-22^T and *Chimaereicella alkaliphila* AC-74^T.

Characteristic	*Chimaereicella boritolerans* sp. nov.		*Chimaereicella alkaliphila**	
	R3V	MB	R3V*	MB
Straight-chain fatty acids:				
$C_{15:0}$	2.63 ± 0.18	0.62	1.60	1.67
$C_{16:0}$	3.53 ± 0.07	3.16	0.40	0.88
$C_{19:0}$	–	–	–	0.50
Branched-chain fatty acids:				
iso-$C_{11:0}$	0.68 ± 0.11	0.17	0.90	0.21
Anteiso-$C_{11:0}$	1.49 ± 0.17	0.43	2.60	1.63
Unknown 13.565	2.13 ± 0.51	1.33	–	1.22
Unknown 13.566	–	–	3.67	–
iso-$C_{14:0}$	0.36 ± 0.01	2.09	0.30	1.37
iso-$C_{15:0}$	21.32 ± 0.13	16.64	32.40	21.01
Anteiso-$C_{15:0}$	6.99 ± 0.81	6.43	3.40	5.14
iso-$C_{15:1}$ G	0.54 ± 0.25	0.22	1.40	0.26
iso-$C_{16:0}$	6.49 ± 0.09	20.66	3.20	11.13
iso-$C_{16:1}$ H	3.29 ± 0.71	10.89	3.80	11.55
Unknown 16.580	–	–	1.30	–
Unknown 16.582	0.64 ± 0.01	0.40	–	0.41
iso-$C_{17:0}$	3.23 ± 0.63	0.77	0.80	0.77

Anteiso-$C_{17:0}$	0.97 ± 0.25	0.29	–	0.18
$C_{17:0}$ Cyclo	–	2.41	–	–
iso-$C_{17:1}$ ω9*c*	13.33 ± 0.74	5.04	14.60	8.01
Anteiso-$C_{17:1}$ ω9*c*	1.88 ± 0.31	1.61	–	0.64
iso-$C_{18:1}$ H	0.64 ± 0.15	1.57	–	0.55
Unsaturated fatty acids:				
$C_{15:1}$ ω 6 *c*	0.28 ± 0.11	–	1.90	1.34
$C_{16:1}$ ω 5 *c*	–	0.47	1.20	2.82
$C_{17:1}$ ω 6 *c*	2.50 ± 0.56	1.30	1.70	1.95
$C_{17:1}$ ω 8 *c*	0.84 ± 0.08	0.29	0.40	0.39
$C_{18:1}$ ω 5 *c*	0.53 ± 0.21	0.71	–	–
Hydroxy fatty acids:				
2-OH $C_{15:0}$	0.63 ± 0.02	0.14	0.50	0.27
2-OH $C_{17:0}$	2.06 ± 0.13	1.09	–	0.71
3-OH iso-$C_{15:0}$	2.20 ± 0.11	0.55	3.20	1.35
3-OH iso-$C_{16:0}$	0.95 ± 0.02	4.48	1.30	2.95
3-OH iso-$C_{17:0}$	7.46 ± 0.33	4.93	7.40	4.64
Summed features[†]:				
3	9.99 ± 0.65	8.94	9.20	12.79
4	0.44 ± 0	–	3.90	1.14

* Data from Tiago *et al.* (2006).

[†] Summed features 3 and 4 are comprised of the group of fatty acids $C_{16:1}$ ω7*c* / iso-$C_{15:0}$ 2-OH, and iso-$C_{17:1}$ I / anteiso-$C_{17:1}$ B, respectively, which cannot be separated reliably by the MIDI system.

Only fatty acids comprising >0.50% of at least one of the strains are included.

G+C content and DNA-DNA hybridization

The genomic DNA of the strain was isolated from cells grown on agar plates according to the method of Marmur (1961), with the slight modification of the use of RNase T_1 in addition to RNase A. The DNA G+C content was determined as described previously (Ahmed *et al.,* 2007a). The DNA G+C content of strain T-22^T was 42.5 mol%.

For DNA–DNA hybridization experiments, Qiagen Genomic-tips 500/G (Qiagen, Germany) were used to isolate the genomic DNA of the novel strain and the closely related type species 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-labeled DNA using micro plates, as described by Ezaki *et al*. (1989). The DNA–DNA relatedness value of strain T-22^T was 28.3% with *C. alkaliphila* LMG 22694^T, thus indicating that strain T-22^T is a novel species (Stackebrandt & Goebel, 1994).

Discussion

Strain T-22^T shared many common and differentiating characteristics with the closely related species *C. alkaliphila* (Table 5.1), and additional characteristics are included in the description of *Chimaereicella boritolerans* sp. nov. It differed from the type strain of the genus, *C. alkaliphila*, which cannot grow at neutral pH (Tiago *et al.,* 2006). No growth of strain T-22^T was observed at temperatures of 10ºC or 40ºC. This feature also differentiates the strain from the closely related species *C. alkaliphila* and the strains of genera *Algoriphagus* and *Hongiella*, which can grow at ≤10ºC (Bowman *et al*., 2003; Nedashkovskaya *et al.*, 2004; Van Trappen *et al*., 2004; Yoon *et al.*, 2004; Tiago *et al.*, 2006). Strain T-22^T survived on agar medium containing up to 300 mM B, whereas the closely related strain *C. alkaliphila* could not tolerate 100 mM B. In addition to B tolerance, the major differentiating characteristics of strain T-22^T from its closely related type strain *C. alkaliphila* were its negative results for gelatin hydrolysis and nitrate reduction tests and sensitivity to cephalothin and chloramphenicol antibiotics.

The fatty acid profile of strain T-22^T was similar to that of *C. alkaliphila* (Table 5.2). Strain T-22^T produced a significant amount of *β*-hydroxy (3-OH) fatty acid components of i-17:0, i-16:0, and i-15:0 (Table 5.2), which are commonly found among members of the Flavobacteria (Bowman *et al*., 1998). The strain also contained an *α*-hydroxy (2-OH) fatty acid, which is absent from the fatty acid profile of *C. alkaliphila. Chimaereicella alkaliphila* has a DNA G+C content of 43.5 mol%, which was slightly higher than that of the novel strain. However, the highest 16S rRNA gene sequence similarity occurred between strain T-22^T and *C. alkaliphila* (Figure 5.1). The critical analysis of other physiological (Table 5.1)

and chemotaxonomic (Table 5.2) data also suggests that strain T-22^T belongs to the genus *Chimaereicella*.

The phylogenetic analysis based on the 16S rRNA sequence, the DNA base composition, and the chemotaxonomic analyses clearly suggest that strain T-22^T belongs to the genus *Chimaereicella.* However, the DNA–DNA homology and morphological and phenotypic characteristics (i.e., pH range for growth, B tolerance, and gelatin hydrolysis and nitrate reductase activity) suggest that it is a novel species in the genus *Chimaereicella*. We propose the name *Chimaereicella boritolerans* sp. nov. for type strain T-22^T described here.

Description of *Chimaereicella boritolerans* sp. nov.

Chimaereicella boritolerans (bo.ri.to.le.rans. N.L. n. *borum*, boron; L. part. adj. *tolerans* tolerating; N.L. part. adj. *boritolerans* boron-tolerating).

Cells of strain T-22^T are non-motile and rod-shaped, 1.2–3.4 μm in length and 0.3-0.4 μm in diameter, occurring singly and occasionally in pairs. Gram staining is negative. Colonies are red-pigmented, circular with entire margins, convex, butyrous in texture, and small in diameter after several days of growth at 30ºC. The red pigment loses its intensity at pH 10.0 on agar medium and is diffusible in liquid medium. The strain grows optimally at 28–30ºC; there is very slight growth at 37ºC and at 16ºC after several days. The optimum pH for growth is 8.0–9.0, with a range of 6.5–10.0. It can tolerate up to 300 mM B, but grows optimally without B supply. The NaCl salt tolerance range is up to 3% (w/v). Oxidase, catalase, hydrolysis of esculin, and *β*-galactosidase tests are positive, whereas production of indole, NO_3 reduction, and hydrolysis of gelatin and urea tests are negative. The strain produces acid from the following substrates: D-galactose, D-glucose, esculin, inulin, D-rafinose, amidon (starch), D-cellobiose, D-maltose, D-lactose, potassium 5-ketogluconate, gentibiose, D-turanose. It can use the following substrates as a carbon source: D-mannitol, amygdalin, arbutin, esculin, inulin, and D-melezitose. Additional physiological characteristics are provided in Table 5.1. Major cellular fatty acids are: iso-

$C_{15:0}$, iso-$C_{17:0}$ ω9*c*, iso-$C_{15:0}$ 2-OH or $C_{16:0}$ ω7*c*, iso-$C_{17:0}$ 3-OH, iso-$C_{16:0}$, and traces of some other types. The respiratory lipoquinone system is MK-7. Strong enzyme activity is observed for alkaline phosphatase, α-chymotrypsin, *N*-acetyl-β-glucosaminidase, and valine arylamidase, whereas weak activity is observed for leucine arylamidase, naphthol-AS-BI-phosphohydrolase, esterase (C8), esterase lipase (C8), trypsin, acid phosphatase, *β*-galactosidase, and α-glucosidase (API-ZYM strip). The strain is resistant to kanamicin, oxacillin, sulfamethizol, and metronidazol, and weakly sensitive to penicillin, gentamicin, rifampicin, and cefoperazon (ATB-VET strip). The G+C content of the strain is 42.5 mol%.

Strain T-22^T (DSM 17298^T = NBRC 101277^T = ATCC BAA-1189^T) is the type strain of *Chimaereicella boritolerans* sp. nov., isolated from soil naturally containing B (Hisarcik, Kutahya, Turkey).

Acknowledgments

We are thankful to Dr. Cavit Çöl for collecting soil samples from the Hisarcik area (Turkey), Dr. I. Tiago for providing his strain, and JEOL (Japan) for their help with the electron microscopy.

CHAPTER VI

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

ABSTRACT

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

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* The DDBJ/EMBL/GenBank accession numbers for the 16S rDNA sequences of strains 10a^T (DSM 17140^T = IAM 15262^T = ATCC BAA-1146^T), 11c, and 12B are AB199591, AB199592, and AB199593, respectively.

INTRODUCTION

Boron is an essential micronutrient for plants (Warington, 1923) and possibly 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. Due to its toxic effects at high concentrations for microorganisms, boron has been used as a food preservative (Nielsen, 2004), and also as an insecticide against cockroaches (Cochran, 1995). However, only recently, *Bacillus boroniphilus, Chimaereicella boritolerans* and *Gracilibacillus boraciitolerans* have been reported to tolerate more than 450, 300 and 450 mM boron, respectively, and/or requires boron for growth (Ahmed *et al.*, 2007a, b & c).

During the last decade, the genus *Bacillus* was taxonomically dissected 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; Yoon *et al.*, 2002). The 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); however, no proposal has yet been put forward to transfer them into one or several new or existing genera. In this paper, we characterize a new round-spore-forming species with a proposed name of *Lysinibacillus boronitolerans* gen. nov. sp. nov. in new genus *Lysinibacillus* gen. nov. based on the phylogenetic and chemotaxonomic characteristics in the *Bacillus* RNA group 2.

This genus is distinguished from other members of this group by the presence of lysine and aspartate in the peptidoglycans of cell wall.

Isolation

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 (i.e. strains $10a^T$, 11c and 12B) were maintained on agar medium; also stored in glycerol (35%, w/v) stock at –80ºC and subjected to characterization.

Boron-tolerance assay

To demonstrate boron tolerance, the strain $10a^T$ and *Escherichia coli* (control) were grown up to the upper mid-log phase (OD_{600} 1.2) at 37ºC, with vigorous shaking. The culture was serially diluted and spotted (7 µL) on nutrient agar (NA, Difco) medium (pH 7.0) containing different boron levels. The plates were incubated at 37ºC for 2 days before being photographed. The strain $10a^T$ manifested tolerance to boron up to 100 mM on nutrient agar medium, whereas weak growth was observed at 150 mM boron (Figure 6.1). The strain was also cultured in 150 mL LB medium (pH 7.0) with different boron levels ranging from 0 (control) to 300 mM boron, while being vigorously shaken at 30ºC. After every 60 min, samples were taken aseptically to measure OD_{600} using a spectrophotometer (Hitachi U-1800). The data for OD_{600} vs. time plotted at different boron concentrations demonstrated that the strain $10a^T$ could grow between 0 and 100 mM boron in liquid culture (LB medium), and the growth was equivalent to that without addition of boron (Figure 6.2), indicating that boron was not required for the growth. However, no growth was observed at 200 mM boron and a weak growth at 150 mM boron.

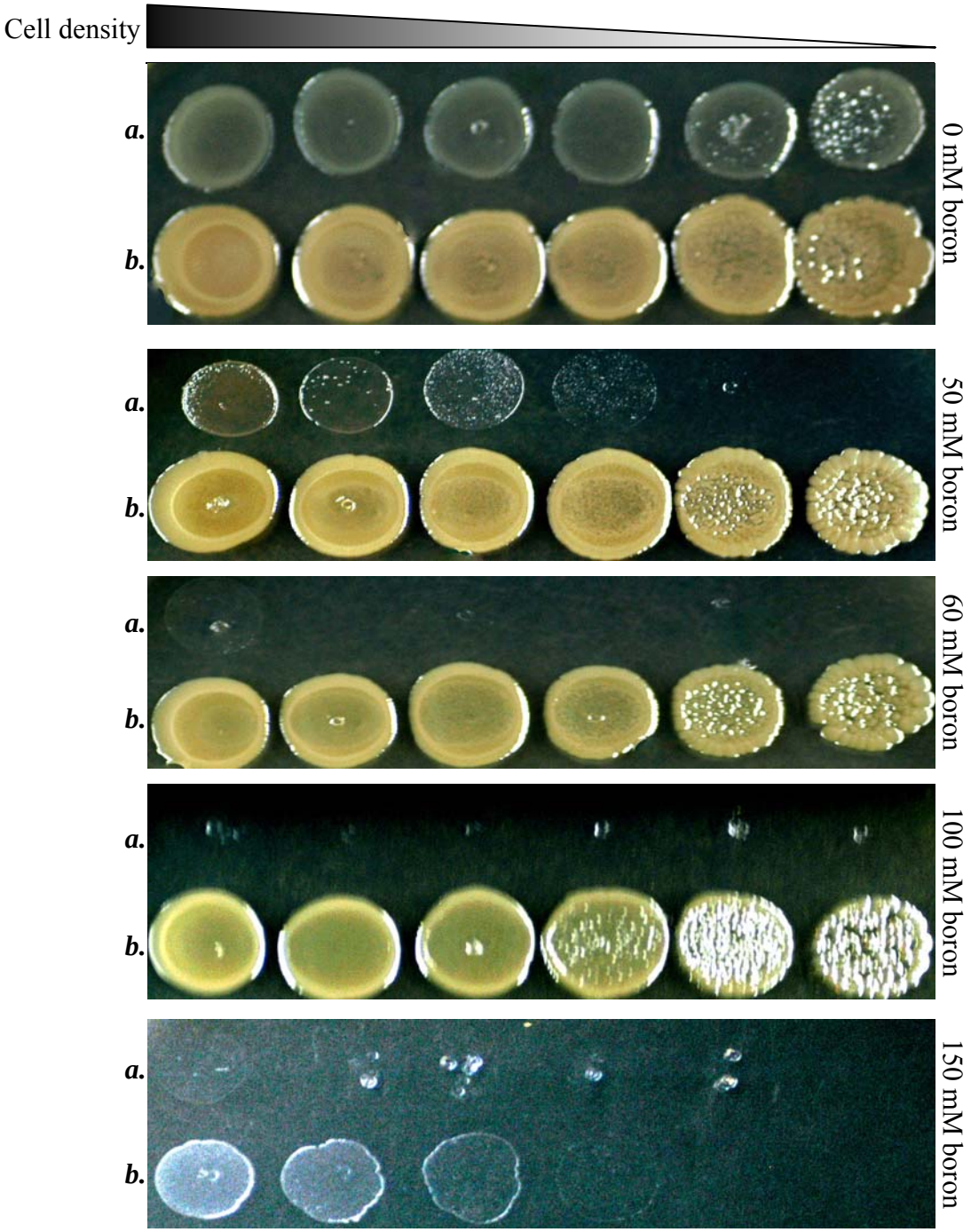


Figure 6.1.

Growth of *Escherichia coli* (a) and strain $10a^T$ (b) on nutrient agar medium (pH 7.0) with different concentrations of boron. The strains grown in Luria–Bertani (LB) medium (pH 7.0) up to upper mid-log phase (OD_{600} 1.2) at 37ºC were serially diluted and spotted (7 µL) on nutrient agar medium (pH 7.0) containing different B levels. The plates were incubated for 2 days at 37ºC and photographed.

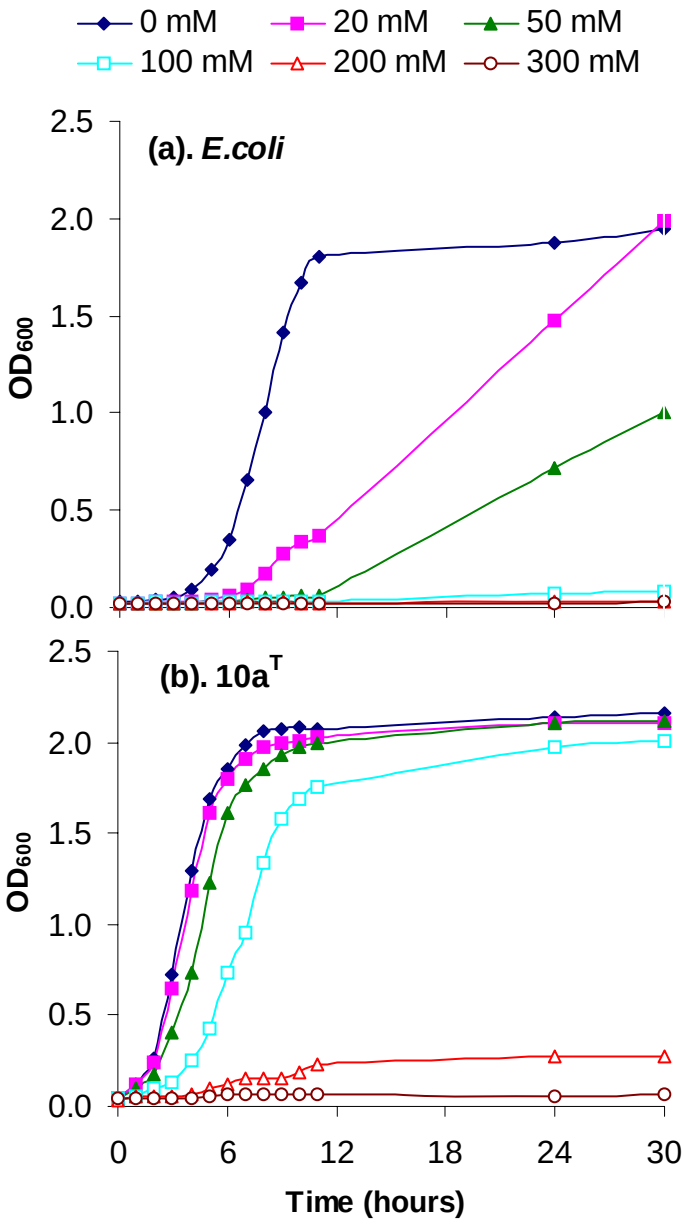


Figure 6.2.

Comparison of the growth curves for (a) *Escherichia coli* (control) and (b) strain $10a^T$ at different boron levels (mM) in Luria–Bertani (LB) medium (pH 7.0). Lines representing the data at high boron levels (100, 200, and 300 mM) in (a) are overlapping.

Morphology

Sporangia and the sizes of cells grown on nutrient agar (Difco) with $MgSO_4.7H_2O$ (1.01 mM), KCl (13.4 mM), $FeSO_4$ (0.001 mM), $Ca(NO_3)_2$ (1.0 mM), and $MnCl_2$ (0.01 mM) at pH 7.0 for 6 days at 30ºC, were examined under phase-contrast microscopy. Cells of the strains produced oval or spherical endospores at the terminal position in a swollen sporangium (Figure 6.3). Cells of the strains were Gram-positive as performed according to Hucker's modified method (Cowan, 1974). Colonial morphology was observed on isolated colonies grown on nutrient agar (pH 7.0) for 2 days at 30ºC.

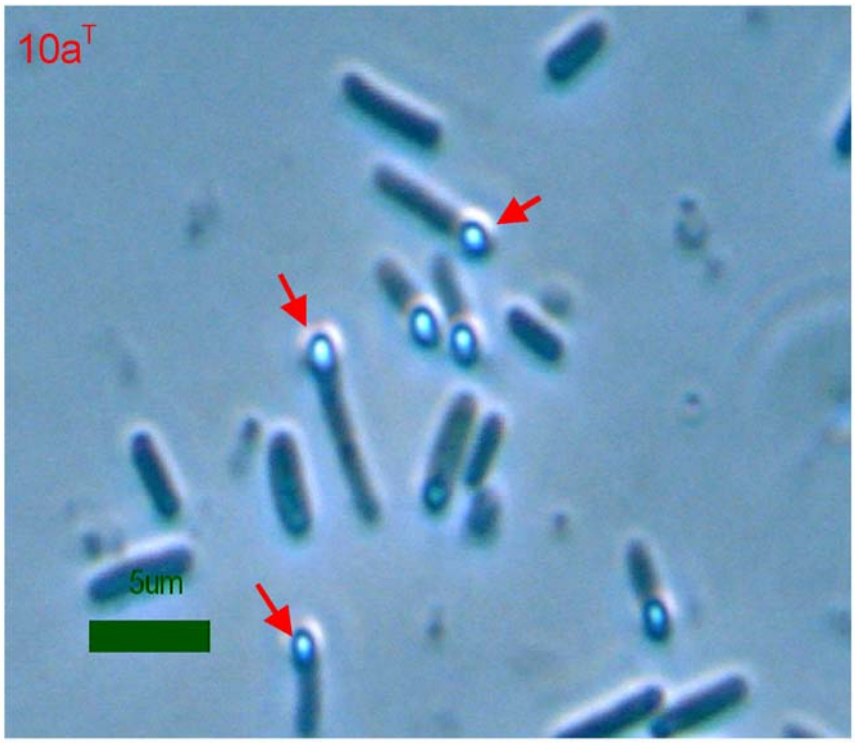


Figure 6.3.

Photomicrograph of strain 10a^T**,** sporangia (indicated by arrows), and vegetative cells viewed under phase-contrast microscopy. The cells were grown on nutrient agar (Difco) medium with $MgSO_4.7H_2O$ (1.01 mM), KCl (13.4 mM), $FeSO_4$ (0.001 mM), $Ca(NO_3)_2$ (1.0 mM), and $MnCl_2$ (0.01 mM), pH 7.0, for 6 days. Bar = 5 µm.

Physiological characteristics

The pH range for growth was determined in tryptic soy broth (with a pH range 4.0 to 10.0) at 30ºC by monitoring OD_{600} using mini-photometer (Model 518R, *TAITEC*, Japan), whereas the temperature range on tryptic soy agar (TSA, pH 7.0, Difco) by incubating at different temperature conditions (from 16ºC to 50ºC). The isolated strains grew at a pH range of 5.5 to 9.5, with an optimum pH of 7.5, but no growth was observed at pH 5.0. We observed the growth of strains in a temperature range of 16 to 45ºC, with an optimum at 37ºC; no growth occurred at ≥50ºC and only slight growth was observed after several days at 16ºC. These findings distinguished the strains from the closely related species, *Bacillus fusiformis* and *B. sphaericus*, which can grow only up to 40ºC. Growth at various NaCl concentrations was investigated on TSA (pH 7.0) at 30ºC. The novel strains tolerated 0–5% (w/v) NaCl in the agar medium. This differed from *Bacillus 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 salt.

Physiological and biochemical characteristics were determined using API 20E and API 50CH galleries (bioMérieux). The strains were positive for oxidase and catalase activities as performed 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 the API system (API 50CH and API 20E) for utilization of various carbon sources, we analyzed an extended array of metabolic features of the strains using the BIOLOG GP2 and GN2 characterization system (BIOLOG). Resistance to antibiotics was assessed by an ATB–VET strip (bioMérieux), while enzyme activity was 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 *Bacillus fusiformis* and *B. sphaericus*; however, it differed from these type strains in certain physiological and biochemical characteristics (Table 6.1). All the three strains (i.e. $10a^T$, 11c and 12B) of the novel species were found identical in these tests.

Table 6.1. Characteristics that differentiate the *Lysinibacillus boronitolerans* gen. nov. sp. nov. from the most closely related species.

1. *Lysinibacillus boronitolerans* gen. nov. sp. nov. ; **2.** *Bacillus fusiformis* DSM 2898^T (*Lysinibacillus fusiformis*); **3.** *Bacillus sphaericus* IAM 13420^T (*Lysinibacillus sphaericus*); **4.** *Bacillus odysseyi* ATCC PTA-4993^T; **5.** *Bacillus massiliensis* CIP 108446^T; **6.** *Bacillus silvestris* DSM 12223^T; **7.** *Bacillus pycnus* JCM 11075^T; **8.** *Bacillus neidei* JCM 11075^T; **9.** *Bacillus arenosi* LMG 22166^T; and **10.** *Bacillus arvi* LMG 22165^T. All type strains produced positive results for motility, growth without NaCl and catalase, and negative results for H_2S production, and indole production. **Symbols:** B, bulging; C, central; O, oval or slightly oval; R, round; T, terminal; nd, data not determined; V, variable results; +, >85% positive; +/w, positive or weakly positive; w, weak positive; –, negative reaction.

Characteristics	1*	2#	3⚙	4‡	5†	6♦	7§	8§	9**	10**
Cellular morphology:										
Size (µm) (length X diameter)	3.0–5.0 X 0.8–1.5	nd	1.5–5.0 X 0.6–1.0	4.0–5.0 X 1.0	1.5–4.0 X 0.3–0.5	0.9–2.0 X 0.5–0.7	3.0–5.0 X 1.0–1.5	3.0–5.0 X 1.0	3.0–8.0 X 0.8–1.0	3.0–8.0 X 0.8–1.0
Spore shape & 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 at:										
Temp. 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:										
NaCl (7%, w/v)	–	+	–	–	–	–	–	nd	–	–
Boron (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 or L-ornithine decarboxylase	–	nd	nd	+	+	nd	nd	nd	–	–
Tryptophane deaminase	+	nd	–†	–†	+	–†	–†	–†		
Hydrolysis of:										
Urea	+	+	–	–	+	+†	–†	+†	–	+
Gelatin	–	+	+	–	–	–	+†	+†	+w	–

Aesculin	+w	–	–	nd	nd	–	nd	nd	–	–
Acid production from:										
D-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
Tetracyclin (2)	–	–	+	nd	–	nd	–	–	nd	nd
Oxidation of:										
Pyruvate	+	+‡	+‡	+	+	+†	+	–	nd	nd
α-Hydroxybutyrate	+	–‡	–‡	+	–	–†	–†	nd	nd	nd
β-Hydroxybutyrate	–	–‡	–‡	+	–	+†	+	–	nd	nd
L-alanine	+	+‡	+‡	+	–	+†	–	–	nd	nd
Glycyl L-glutamate	+	+‡	+‡	+	–	+†	–	+	nd	nd
Adenosine	+	+‡	+‡	–	–	+†	+†	+	nd	nd
2'-Deoxyadenosine	+	+‡	+‡	+	–	+†	–	+	nd	nd
Inosine	+	+‡	+‡	+	–	+†	–	+	nd	nd
AMP	+	+‡	+‡	+	–	+†	–	+	nd	nd
UMP	+	+‡	+‡	+	–	–†	–	+	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

* Data from present study.
# Data from Priest *et al.* (1988)
⊙ Data from Claus & Berkeley (1986)
‡ Data from La Duc *et al.* (2004).
† Data from Glazunova *et al.* (2006).
⇑ Data from Rheims *et al.* (1999)
§ Data from Nakamura *et al.* (2002)
** Data from Heyrman *et al.* (2005)

Table 6.2. Characteristics that differentiate the genus *Lysinibacillus* gen. nov. from the closely related genera.

Character	*Lysinibacillus*	*Caryophanon* *	*Kurthia* †	*Sporosarcina* *	*Bacillus* ‡
Endospore	+	–	–	+	+
Rod-shaped	+	Trichome rods	+	–	+
Oxidase	+	–	na	+	+
Nitrate reduced to nitrite	–	–	–	+	+
Cell wall type	Lys-Asp	L-Lys-D-Glu[§]	L-Lys-D-Asp	L-Lys-Gly-D-Glu[§]	*m*-DAP[#]
Menaquinone system	MK-7	MK-6	MK-7	MK-7	MK-7
Major Fatty acid	Iso-$C_{15:0}$	na	na	Anteiso-$C_{15:0}$**	Anteiso-$C_{15:0}$‡‡
G+C content (mol%)	35–38 ۞	44–46	36–38	40–42	32–69

Symbols: +, positive; –, negative reaction; na, data not available.
* Data from Claus, Fritze & Kocur (1992).
† Data from Keddie & Jones (1992).
‡ Data from Claus & Berkeley (1986).
§ Data from Stackebrandt *et al.* (1987).
# Data from Schleifer & Kandler (1972).
**Data from Yoon *et al.* (2001)
‡‡Data from Kämpfer *et al.* (2006)
۞ Data for G+C content of *Bacillus fusiformis* from Fahmy *et al.* (1985) and *B. sphaericus* from Priest *et al.* (1988).

16S rRNA sequencing and phylogenetic analysis

Nearly complete 16S rRNA gene sequences of the strains were amplified by the polymerase chain reaction (PCR) as described by Katsivela *et al.* (1999). The purified PCR product was sequenced as previously described (Ahmed *et al.*, 2007a). The DNASIS Pro (Hitachi Software Engineering) software package was used to obtain the consensus sequence. The sequence data of the closely related type strains used for constructing phylogenetic tree were selected and retrieved from the DNA Data Bank of Japan/European Molecular Biology Laboratory (DDBJ/EMBL) databases by BLAST searches for bacteria. Alignment was performed with CLUSTAL X (ver. 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 neighbor-joining method (Saitou & Nei, 1987) contained in PHYLIP software package (Felsenstein, 2005) and plotted with NJ Plot 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 to the 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 found to be 97.2% with *Bacillus fusiformis* (AJ 310083), 96.9% with *B. sphaericus* (AJ 310084), 96.1% with *B. odysseyi*, 95.0% with *B. massiliensis*, and 94.5% with *B. silvestris* (AJ 006086); 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 data of strain 11c was omitted during construction of the phylogenetic tree due to incomplete sequences. The novel strains occupied a separate lineage in the phylogenetic tree with high bootstrap support (Figure 6.4). Strain $10a^T$ is closely related to *Bacillus fusiformis* and *B. sphaericus*

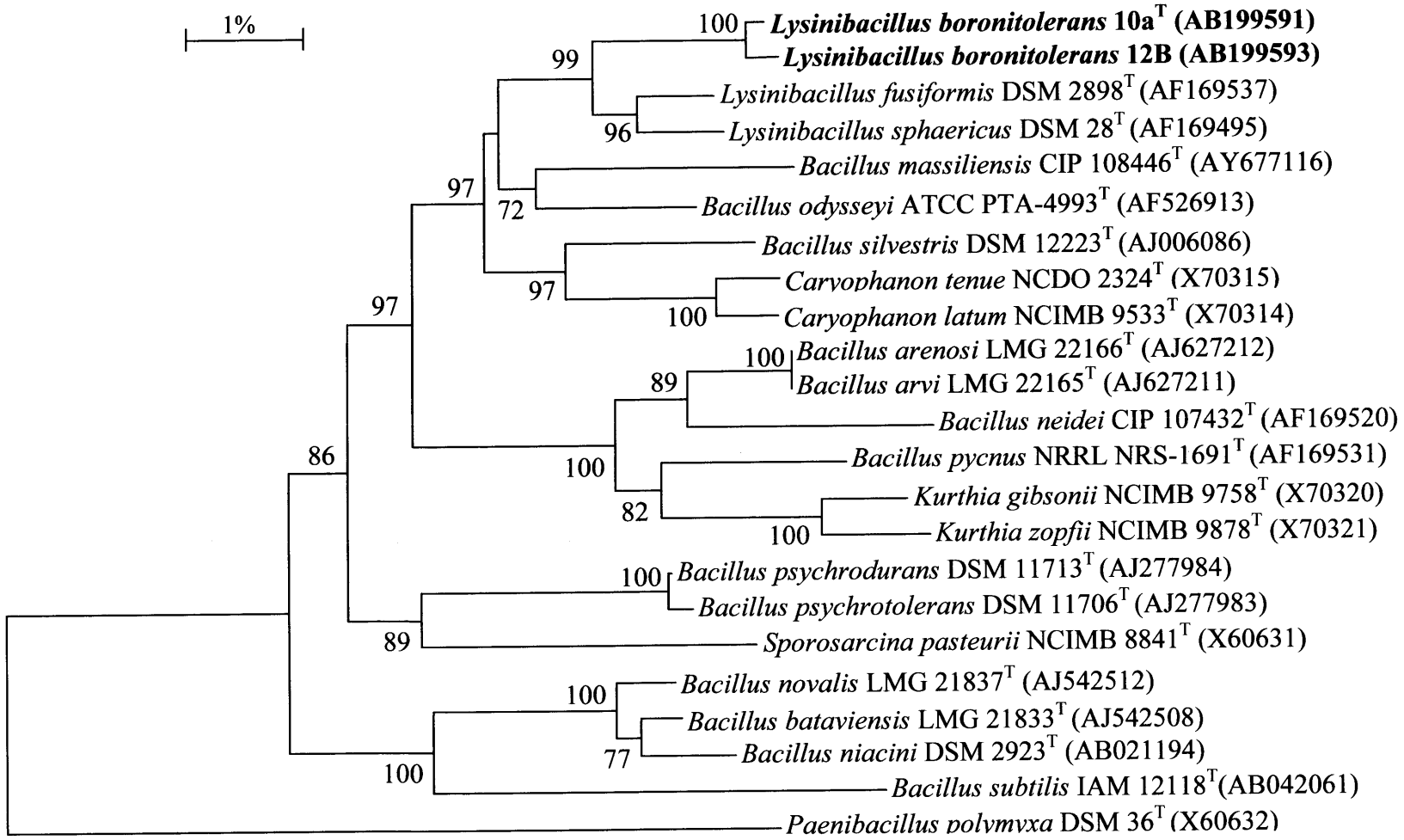


Figure. 6.4.

Phylogenetic tree showing the interrelationships among the strains (10a^T and 12B) of *Lysinibacillus boronitolerans* gen. nov. sp. nov. and their close relatives inferred from 16S rDNA sequences. Data with gaps were removed using BioEdit software during alignment for constructing the rooted-tree using the **neighbor-joining method** contained in PHYLIP package (Felsenstein, 2005) and plotted with NJ Plot, based on a comparison of approximately 1294 nucleotides. Bootstrap values, expressed as a percentage of 1000 replications, are given at the branching points. The sequence of *Paenibacillus polymyxa* DSM 36^T (X60632) was used as the outgroup. Bar = 1% sequence divergence.

in many other characteristics, although the data presented here also exhibited differences from these and other type strains of the genus *Bacillus* (Table 6.1) and among closely related genera (Table 6.2). On the basis of the morphological, physiological, phylogenetic, chemotaxonomic and genomic characteristics, which we determined, the strains 10a^T, 11c and 12B were considered as members of the same species to be proposed as *Lysinibacillus boronitolerans* gen. nov. sp. nov.

DNA-DNA hybridization and G+C content

For the DNA–DNA hybridization experiment, DNA from the isolated strains and the closely related species were isolated using a previously described procedure (Ahmed *et al*., 2007a). The DNA–DNA hybridization was performed at 42°C with photobiotin-labeled 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 the three strains (i.e. 10a^T, 11c and 12B) were more than 97.8%, to conclude that these strains belonged to the same species. However, DNA–DNA relatedness of strain 10a^T was 61.1% with *Bacillus 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 describe the strains as 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 previously described procedure (Ahmed *et al*., 2007a).

Chemotaxonomic analyses

Respiratory quinones were analyzed as described by Xie & Yokota (2003) and MK-7 (87 %) was determined in the novel strains as the predominant quinone system, although MK-6 (13 %) was also detected as a minor component.

The purified cell wall was analyzed for amino acids using two-dimensional TLC and then HPLC (Shimadzu) as described elsewhere (Schleifer & Kandler, 1972; Groth *et al.*

1996). The strain 10a^T contained peptidoglycan with alanine, glutamic acid, lysine, and aspartic acid (Figure 6.5) in the molar ratio of 1.83 : 1.0 : 0.69 : 0.63, respectively as the diagnostic amino acids, in contrast to the type species of the genus *Bacillus*, *Bacillus subtilis* that was diagnosed with *m*-diaminopimelic acid in the cell wall peptidoglycan (Schleifer & Kandler, 1972). During this study, *Bacillus fusiformis* also produced similar results for cell wall peptidoglycan analysis as lysine, alanine, glutamic acid and aspartic acid in the same molar ratio as for the strain 10a^T. This represents peptidoglycan type A4α (Lys-Asp), as described by Schleifer & Kandler (1972). Also the close type relative, *Bacillus sphaericus* was reported to contain with L-Lys-D-Asp in the peptidoglycan as the diagnostic amino acids (Stackebrandt *et al.*, 1987). So far a peptidoglycan consisting of Lys–Asp has not been reported for any other endospore–forming species of the *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* (Figure 6.4) are sharing this characteristic (Shaw and Keddie, 1983).

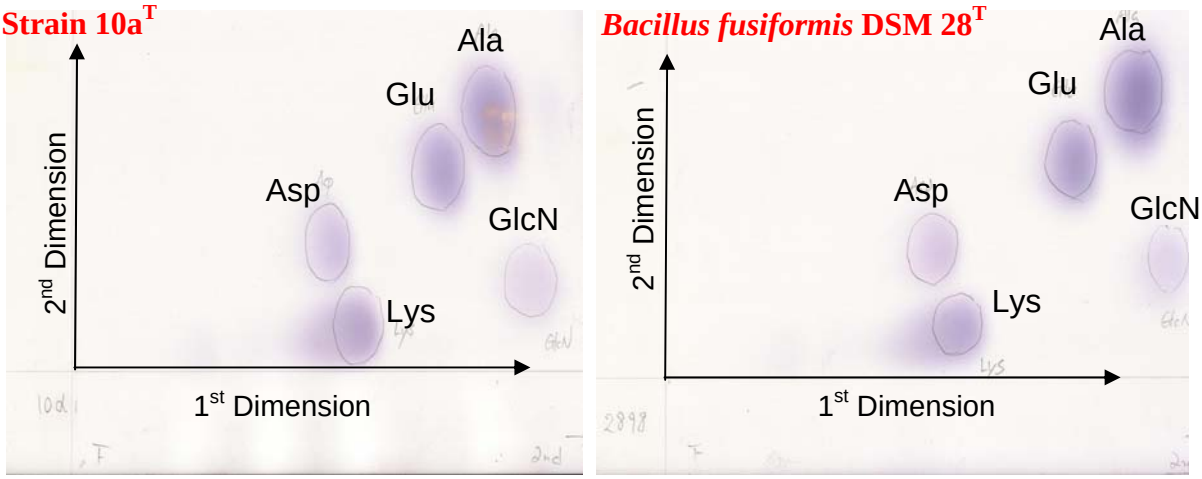


Figure 6.5.

Cell wall peptidoglycan amino acids of the strain 10a^T (a) and *Bacillus fusiformis* DSM 28^T (b) as analyzed by using two-dimensional thin-layer chromatography (2-D TLC).

Fatty acids analysis

For whole-cell fatty acids analysis, the cells were grown on tryptic soy agar (TSA) for 24 h at 28ºC, 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 species was comprised predominantly of iso– and anteiso–branched fatty acids (Table 6.3) that was similar to other members of the *B. sphaericus*-like group. A MIDI database search of profiles also supported the novel species status, as there was no match to any known species.

Polar lipids analysis

The polar lipids were extracted and purified from 100 mg of dried cells of the strain 10a^T, *Bacillus fusiformis* and *B. sphaericus* by the procedure of Minnikin *et al.* (1984) and examined by 2-D TLC, using Kieselgel 60 F254 plates (E. Merck, Darmstadt, Germany), as described by Kudo (2001). Strain 10a^T shared polar lipids with *Bacillus fusiformis* and *B. sphaericus,* which predominantly consisted of diphosphatidylglycerol, phosphatidylglycerol and ninhydrin-positive phosphoglycolipid (Figure 6.6). The chemotaxonomic data showed some significant differences when compared with the closely related genera, particularly with type species of the genus *Bacillus* in terms of polar lipids analysis (Kämpfer *et al.,* 2006).

On the basis of chemical composition, genotypic and phenotypic data presented in this paper, the isolated strains (10a^T, 11c and 12B) are proposed as a new species in a new genus *Lysinibacillus* gen. nov. sp. nov. with the type strain 10a^T. The chemotaxonomic data and phylogenetic analyses (Figure 6.4) also demonstrated that *Bacillus fusiformis* and *B. sphaericus* should be transferred to the genus *Lysinibacillus* as *Lysinibacillus fusiformis* comb. nov. and *Lysinibacillus sphaericus* comb. nov., respectively. Data on peptidoglycan composition and polar lipids of the next phylogenetically-related species, *Bacillus massiliensis* and *B. odysseyi* are not available; therefore, inclusion of these species in the novel genus *Lysinibacillus* gen. nov. is not recognized here. *Bacillus silvestris* contains peptidoglycans with L-lysine and D-glutamate, whereas major polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, along with minor amounts of phosphatidylserine and one unknown phospholipid. This chemical composition differentiate *Bacillus silvestris* from the novel genus *Lysinibacillus* gen. nov.

Table 6.3. Cellular fatty acid composition (%) of *Lysinibacillus boronitolerans* gen. nov. sp. nov. and the closely related taxa.

1. *Lysinibacillus boronitolerans* gen. nov. sp. nov. ; **2.** *Bacillus fusiformis* DSM 2898^T (*Lysinibacillus fusiformis*) ; **3.** *Bacillus sphaericus* IAM 13420^T (*Lysinibacillus sphaericus*) ; **4.** *Bacillus arenosi* LMG 22166^T and **5.** *Bacillus arvi* LMG 22165^T, data from Heyrman *et al.* (2005); **6.** *Bacillus massiliensis* CIP 108446^T, data from Glazunova *et al.* (2006); **7.** *Bacillus silvestris* DSM 12223^T, data from Rheims *et al.* (1999).

Characteristics	**1** n = 6	**2** n = 3	**3** n = 3	**4** n = 3	**5** n = 2	**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-$C_{17:1}$ ω10*c*	1.3 ± 0.29	0.9 ± 0.02	8.3 ± 0.9	4.9 ± 0.1	1.3 ± 0.1	–	7.8[a]
$C_{16:1}$ ω7*c* alcohol	7.6 ± 0.90	11.6 ± 0.3	18.3 ± 2.7	4.3 ± 1.2	10.7 ± 1.2	2.6	3.1[b]
$C_{16:1}$ ω11*c*	2.7 ± 0.52	5.8 ± 0.2	3.3 ± 0.07	7.3 ± 0.8	6.1± 0.4	0.7	–
$C_{18:1}$ ω9*c*	–	–	–	–	–	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	–	–

The summed feature 4 comprises of iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.

The results of *Lysinibacillus boronitolerans* gen. nov. sp. nov. are based on two replicates for each strain.

[a] & [b]: In the original paper (Rheims *et al.*, 1999), these fatty acids were designated 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*).

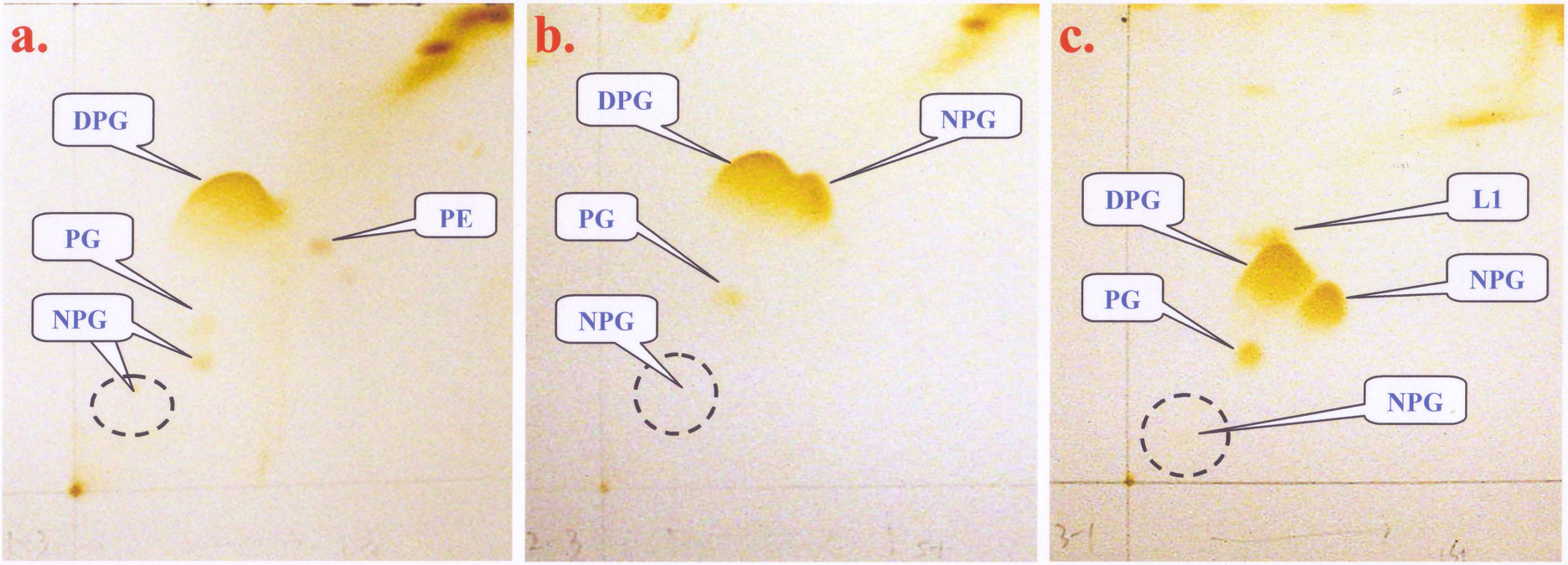


Figure 6.6.

Polar lipids profiles after separation by two-dimensional thin-layer chromatography. **(a).** strain 10a^T; **(b).** *Lysinibacillus fusiformis* DSM 2898^T; **(c).** *Lysinibacillus sphaericus* IAM 13420^T. PG, Phosphatidylglycerol; DPG, Diphosphatidyl glycerol; PE, Phosphatidylethanolamine ; NPG, Ninhydrin-positive phosphoglycolipid; L1, unknown polar lipid.

Description of Lysinibacillus gen. nov-

Lysinibacillus (Ly.si.ni.ba.cil'lus. N.L. n. *lysinum*, lysine; L. masc. n. *bacillus*, *baktron* rod, a small staff, a wand; N. L. masc. n. *Lysinibacillus* the bacillus/rod with Lys-Asp type of peptidoglycan cell wall).

Motile and 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 H_2S, NO_3 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 A4α (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 35–43 mol %. The type species of the genus is *Lysinibacillus boronitolerans* sp. nov.

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 those characteristics described for the genus, the novel species, *Lysinibacillus boronitolerans* has the following features. The rod-shaped cells are 3.0–5.0 μm in length and 0.8–1.5 μm 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 nutrient agar (pH 7.0) plate at 37ºC. Temperature range for growth is 16–45ºC, with optimum growth at 37ºC; there is no growth at ≥50ºC and little growth at 16ºC after several days; growth is observed in the pH range 5.5–9.5, with optimum growth at pH 6.5–8.0 (7.5)

but no growth at pH 4.5. It 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 tolerance range is up to 5% (w/v), indicating that it is moderately halotolerant and can grow on Marine Agar 2216 (Difco), TSA, and NA (with or without boron or NaCl). Acid is produced from *N*-acetyl-D-glucosamine, D-xylose (weak), and aesculine (weak) (API 50/CH); however, acid is not produced from carbohydrates (API 20E). It is positive for Voges–Proskauer, urease, L-arginine dihydrolase, tryptophan deaminase and citrate utilization, but negative for hydrolysis of gelatin, L-lysine and L-ornithine decarboxylases and can oxidize 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'-deoxy adenosine, L-serine, pyruvic acid, methyl pyruvic acid, thymidine-5'-monophosphate, mono-methyl succinate, uridine-5'-monophosphate, propionic acid, alaninamide, adenosine, adenosine-5'-monophosphate, lactamide, L-asparagine, thymidine, uridine, α-hydroxybutyric acid, α-keto valeric acid, bromo succinic acid, *cis*-aconitic acid, citric acid, D,L-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%; $C_{16:1}\omega7c$ alcohol, 8%; iso-$C_{17:0}$, 6%; iso-$C_{14:0}$, 2%. Cell wall peptidoglycan contains lysine, alanine, glutamic acid and aspartic acid as the diagnostic amino acids, representing the cell wall peptidoglycan type A4α. In addition to polar lipids given in the genus description, it also contains phosphatidylethanolamine. Strong enzyme activity is observed for α-chymotrypsin and esterase (C8), while weak activity is observed for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, acid phosphatase, valine arylamidase, and naphthol-AS-BI-phosphohydrolase (API ZYM Strip). The strain is resistant to linomycin, colistin, sulfamethizol, oxolinic acid, fusidic acid, and

metronidazol (ATB-VET Strip). 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 of *Lysinibacillus boronitolerans* sp. nov., isolated from soil collected in the experimental area of the University of Tokyo (Yayoi campus), Japan.

Description of *Lysinibacillus fusiformis* comb. nov.

Lysinibacillus fusiformis (fus.i.form'is. L.N. *fusus* spindle; L.N. *forma* shape, form; M.L. adj. *fusiformis* spindle-shaped).

Basonym: *Bacillus fusiformis* Priest *et al.* 1988.

The original description of the species is unchaged as given by Priest *et al.* (1988). Cell wall peptidoglycan contains lysine, alanine, glutamic acid and aspartic acid in the molar ratio of 1.81 : 1.0 : 0.69 : 0.64, respectively as a diagnostic amino acid. Cellular fatty acid profile is listed in Table 6.3. 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. Gr.adj. *sphaericus* spherical; M.L. adj. *sphaericus* spherical-shaped).

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 speices (Table 6.3). 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 = NCIB 9370^T = NCTC 10338^T).

Acknowledgments

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 this Chapter; and the Ministry of Education, Culture, Sports, Science, and Technology of Japan for providing financial assistance to the first author.

CHAPTER VII

Mechanism of Boron Toxicity Tolerance in *Bacteria*

ABSTRACT

We isolated several boron (B) toxicity tolerant bacterial strains from soil. Phylogenetic analysis based on comparative 16S rRNA gene sequence data demonstrated that the isolates belong to six genera; *Rhodococcus, Arthrobacter, Lysinibacillus, Chimaereicella, Gracilibacillus,* and *Bacillus,* exhibiting boron tolerance of 80, 100, 150 300, 450 and 450 mM, respectively, in TSB medium. Analysis of the isolates for boron tolerance revealed that they maintained significantly lower concentration of B in the cells than that in the medium. Statistical analysis of data showed an apparent negative correlation between the protoplasmic boron concentration and the degree of tolerance to a high external boron concentration. These results suggest that efflux and/or exclusion of B is a mechanism of tolerance against high external concentration of B in the isolated bacteria.

Key words:

Bacillus boroniphilus, Lysinibacillus boronitolerans, Boron toxicity, Boron tolerance, Hisarcik area

Abbreviations

B: boron, TSB: tryptic soy broth, PBS: phosphate buffered saline solution

INTRODUCTION

Boron (B) is an essential micronutrient for optimum growth of plants (Warington, 1923) and some animals (Rowe *et al.*, 1998; Rowe and Eckhert, 1999) but can be toxic to organisms when present above a 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 testis 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). However, knowledge of B toxicity tolerance mechanism is limited. 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.

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 isolated and identified microorganisms exhibiting a range of B toxicity tolerance, from two soils: normal soil of greenhouse (Yayoi campus, University of Tokyo) and a naturally high B-containing soil of Hisarcik area in the Kutahya Province of Turkey. The objective of this study was to examine B toxicity tolerance mechanism in the B-tolerant bacteria. Previously, B toxicity tolerance in barley was attributed to lower accumulation of B in both shoots and roots (Nable, 1988; Nable *et al*., 1990). It has been demonstrated that B-uptake was also affected by permeability of membranes to B that could be reduced by altering lipid composition of membranes (Dordos and Brown, 2000 and Dordas *et al*., 2000). Recently, Hayes and Reid (2004) reported an efflux of B as a basis for

tolerance to B toxicity in barley. However, plant tolerance to B toxicity is in the range of few mmol of B, but B-tolerant bacterial strains could grow at a very high B concentration (several hundred mmol of B), and it is possible that more than one mechanism may be responsible for this tolerance. The present report demonstrates the B-tolerance mechanism in these highly B-tolerant bacteria.

MATERIALS AND METHODS

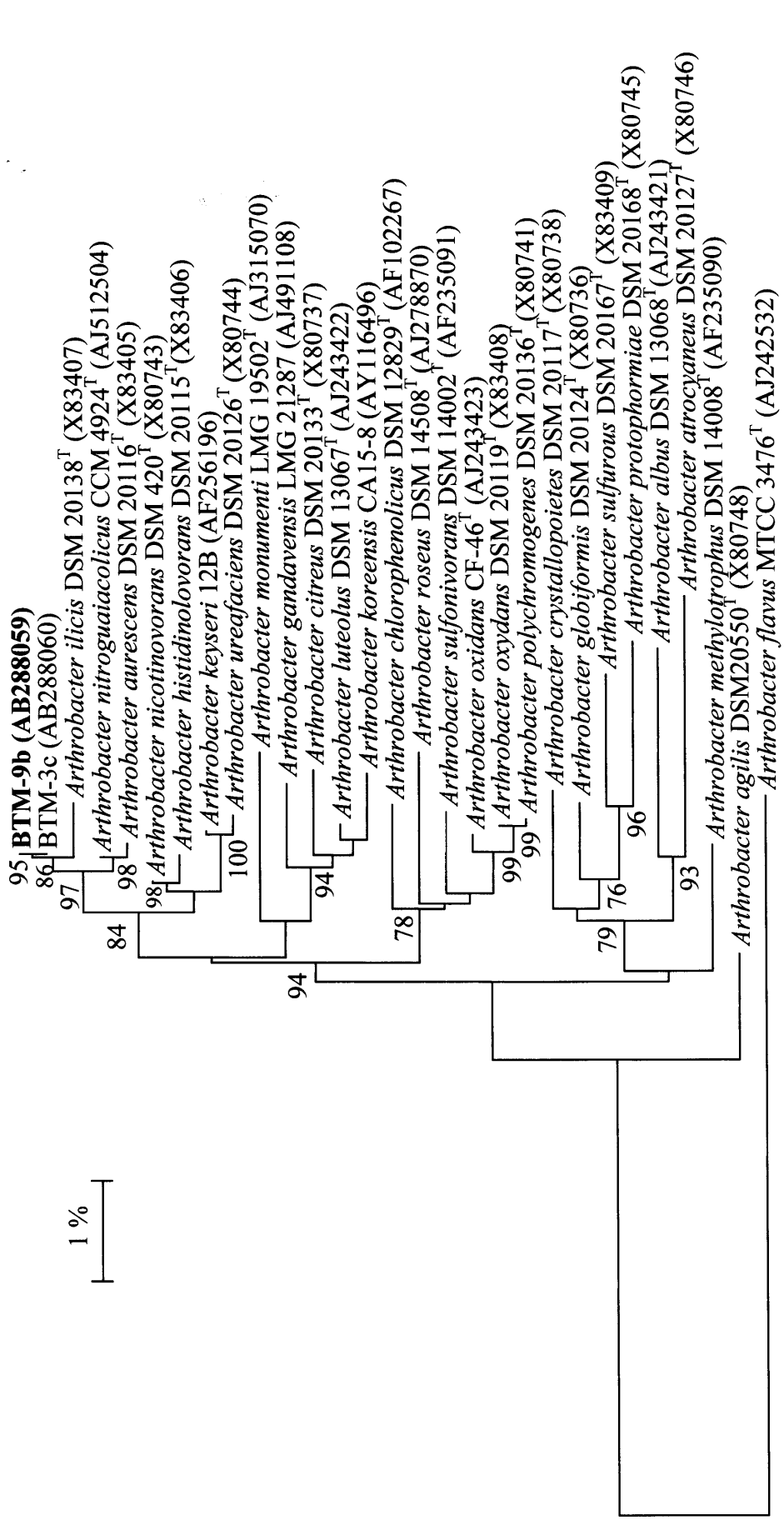
Isolation and identification of the strains

High-B tolerant strains used in this study were isolated from soil of Tokyo (strains: BTM-4b, BTM-9b & 10-a) and Hisarcik area (strain: T-17s), in the Kutahya province of Turkey, as described previously (Ahmed *et. al.,* 2007a). The strains 10-a and T-17s has been characterized and identified as *Lysinibacillus boronitolerans* and *Bacillus boroniphilus* (Ahmed *et al.,* 2007a & b), whereas the strains BTM-9b and BTM-4b belonged to genera *Arthrobacter* and *Rhodococcus*, respectively, having the highest 16S rRNA gene sequence similarity of more than 99.8% with the close cultivated strains in their respective clusters (Supplementary Figure 7.1 & 7.2). The GenBank accession number for 16S rRNA gene sequences of the strains are as follows: BTM-9b *AB288059*, strain BTM-4c *AB288063*, strain 10-a AB199591, strain T-17s *AB198720*.

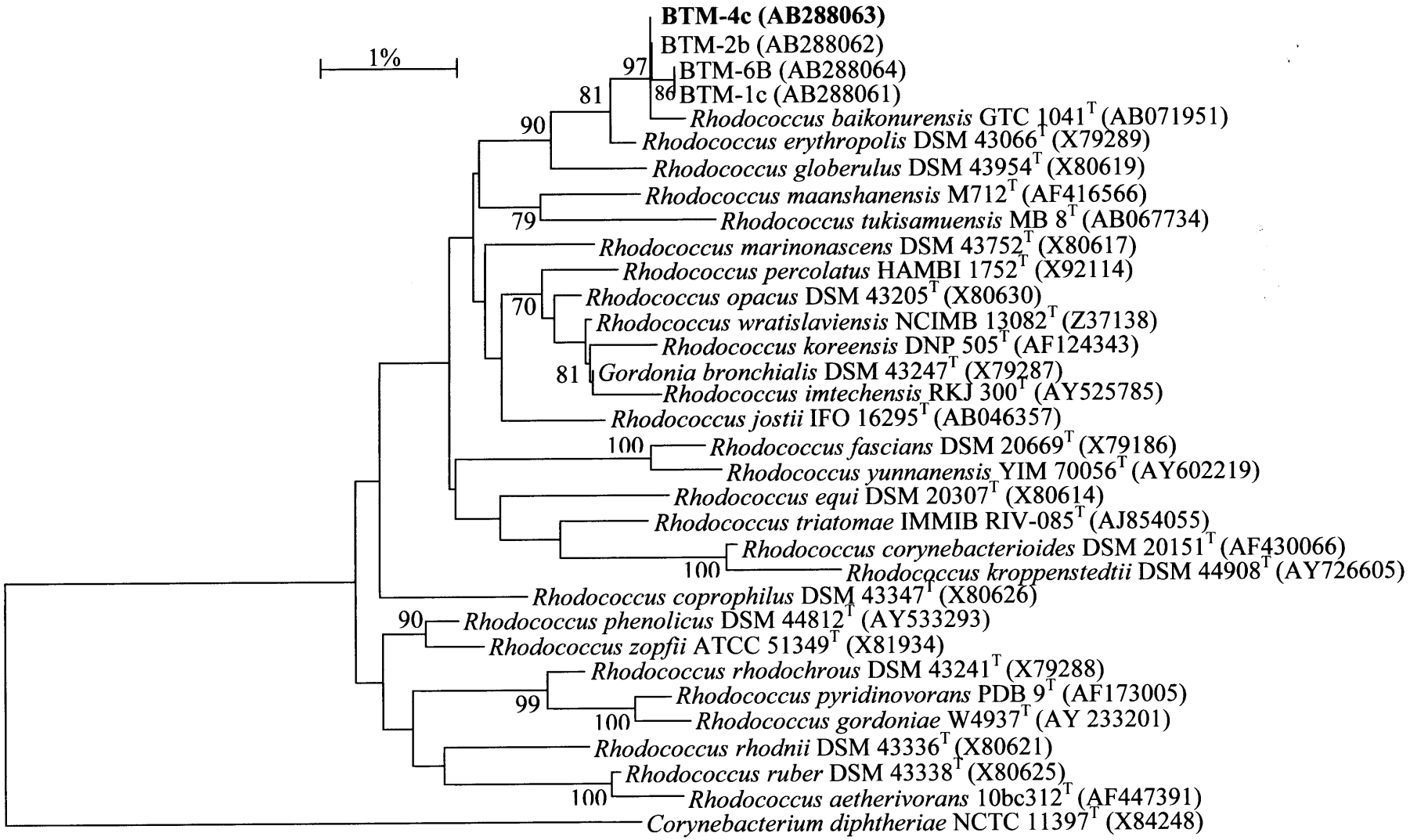
Boron tolerance, uptake and efflux

The B tolerance of the strains was demonstrated according to previously described procedure (Ahmed *et. al.,* 2007a). *Bacillus subtilis* strain ISW 1214 (Takara, Japan) was used as a control.

All the incubations for growth and treatments were carried out at 30 ºC with vigorous shaking. Since strain T-17s cannot grow without B (Ahmed *et al.,* 2007a), the initial growth of cells were obtained in TSB medium (pH 7.4) with 5 mM boric acid enriched with ^{11}B (Cambridge Isotope Laboratories, USA) until upper mid log phase (OD_{600} 0.8~1.0). The harvested cells were then suspended in TSB medium (pH 7.4)



Supplementary Figure 7.1. Phylogenetic tree showing inter-relationship of boron-tolerant strains (BTM-3c and BTM-9b) of the genus ***Arthrobacter*** and its closest relatives inferred from sequences of 16S rRNA gene. Data with gaps were removed during alignment using BioEdit software and the tree was constructed using **neighbor-joining method** contained in PHYLIP software package (Felsenstein, 2005) based on a comparison of approximately 1371 nts and plotted with NJ Plot. Bootstrap values (only more than 70%), expressed as a percentage of 1000 replications, are given at the branching points. The sequence of *Arthrobacter flavus* MTCC 3476^T (AJ242532) is used as the out group. Bar, 1 % sequence divergence.



Supplementary Figure 7.2. Phylogenetic tree showing inter-relationship of boron-tolerant strains (BTM-1c, BTM-2b, BTM-4c and BTM-6B) of the genus ***Rhodococcus*** and its closest relatives inferred from sequences of 16S rRNA gene. Data with gaps were removed during alignment using BioEdit software and the tree was constructed using **neighbor-joining method** contained in PHYLIP software package (Felsenstein, 2005) based on a comparison of approximately 1205 nts and plotted with NJ Plot. Bootstrap values (only more than 70%), expressed as a percentage of 1000 replications, are given at the branching points. The sequence of *Corynebacterium diphtheriae* (X84248) is used as the out group. Bar, 0.5% sequence divergence.

with high (50 mM) and low (10 mM) levels of boric acid enriched with ^{10}B. After one hour of B treatment, cells were harvested by centrifugation (4000g for 2-3 min at 4 ºC) and washed twice with ice-chilled PBS buffer. The pelleted cells were then suspended in deionized water (Milli-Q, Millipore) and were boiled for 30 min. The clarified supernatant and pallet were then taken separately for determination of intracellular soluble and pallet-bound B, respectively.

Strain T-17s, being the most tolerant to toxic levels of boric acid, was further studied for efflux experiment. The cells, grown in TSB medium (pH 7.4) containing 5 mM ^{11}B, were suspended in TSB medium (pH 7.4) containing high (10 mM) and low (0.5 mM) ^{10}B levels for 0, 2, 15, 30, 45, 60 and 90 min. The harvested cells were prepared for B determination as described above. *Bacillus subtilis* being sensitive to B, was used as control.

Boron analysis

For analysis of ^{10}B and ^{11}B, the samples (supernatant for cellular soluble B and freeze-dried cells for pallet-bound B fractions) were digested with 2.5 mL of B-free nitric acid (60~61 %, w/w) in Teflon tubes at 130 ºC to dry completely. The residues were dissolved in 0.08 M HNO_3 containing 5 µg L^{-1} and analyzed by inductively coupled plasma mass spectrometer (ICP-MS, model SI1 SPQ-8000A, Seiko Instruments, Chiba, Japan) with Be as the internal standard.

RESULTS AND DISCUSSION

Fifteen isolated strains were studied for their tolerance to high B levels. The isolated strains showed a wide range of tolerance to toxic B concentrations (Figure 7.1a, auxiliary legend). *Bacillus subtilis*, being the most sensitive, was used as a control that could grow on tryptic soy agar (TSB, Difco) containing 50 mM B but with very poor growth of colonies even after several days of incubation. On the other hand, *Bacillus boroniphilus* tolerated 11-folds more B concentration compared to *Bacillus subtilis* (Figure 7.1a, Auxiliary legend) and could not grow without B in the medium (Ahmed *et al.,* 2007a). The strains belonging

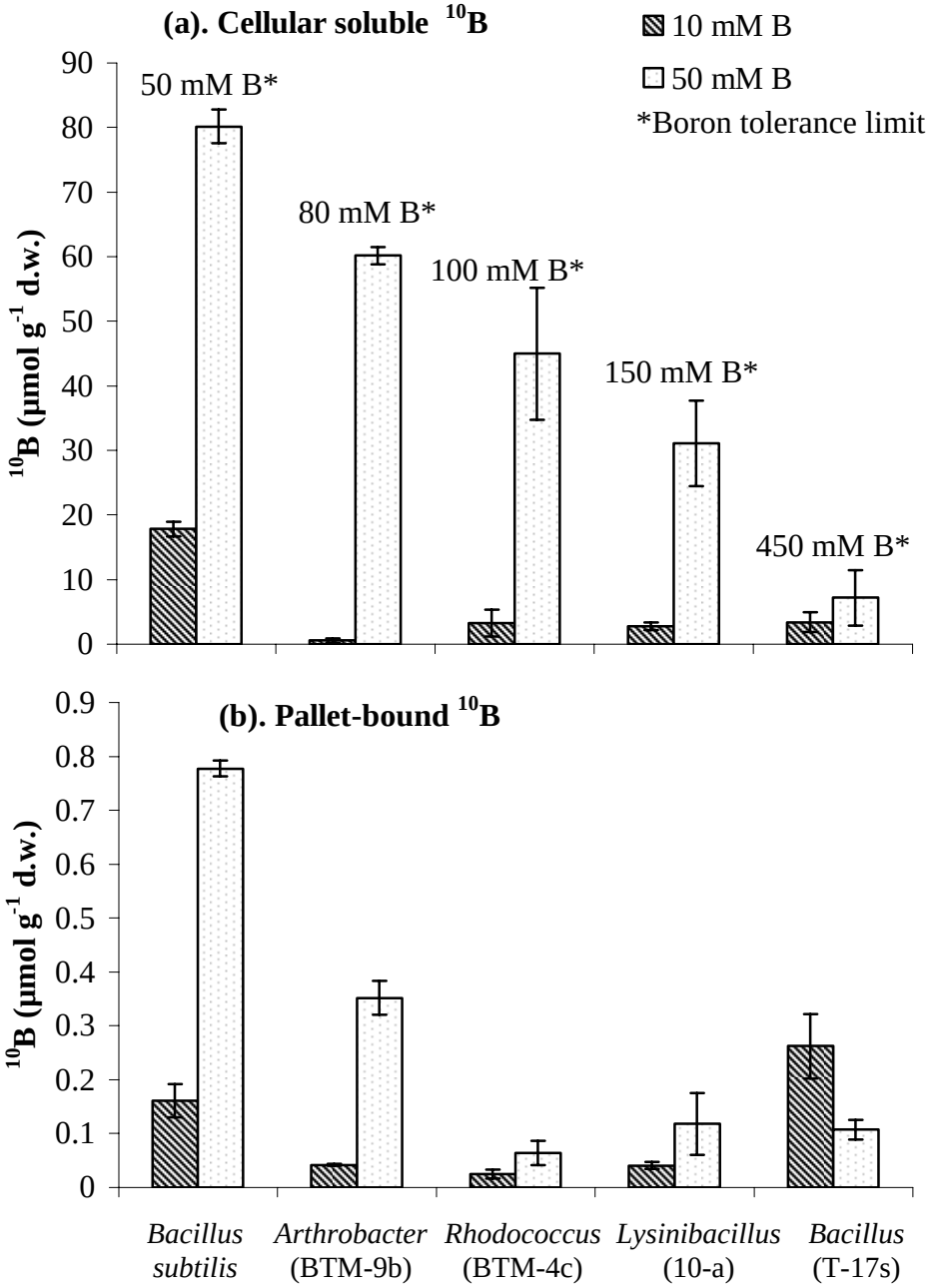


Figure 7.1. Uptake of boron in cells at two levels (10 and 50 mM) of boron supply for one hour. Data are means with error bars (± standard deviation) for four independent replications. d.w., dry weight.

to genera *Rhodococcus, Arthrobacter, Lysinibacillus, Chimaereicella, Gracilibacillus,* and *Bacillus*, exhibited B tolerance of 80, 100, 150 300, 450 (Ahmed *et al*., 2007b) and 450 mM (Ahmed *et al*., 2007a), respectively, in TSB medium. One strain from each B-tolerance limit was further studied for B-uptake experiment. However, being slow in growth, the strains of *Gracilibacillus* and *Chimaereicella* were excluded from B-uptake studies.

At low level of B supply (10 mM ^{10}B), the soluble ^{10}B concentration in the cells of B-tolerant strains was more or less similar compared to B-sensitive strain, *Bacillus subtilis* (control) that accumulated 4-folds more soluble B in the cells (Figure 7.1) that may presumably be governed by membrane lipid composition (Dordos and Brown, 2000 and Dordas *et al*. 2000). However, analysis of cellular soluble B at high level of B supply (50 mM ^{10}B) revealed the clear difference among the isolated strains (Figure 7.1), which was negatively correlated ($r = –0.888$) to the B-tolerance in the medium (Figure 7.2). These results are consistent with the previous reports in plants (Nable *et al.,* 1990; Hayes and Reid, 2004; Reid *et al.,* 2004). The difference of intracellular soluble B at low (10 mM) and high (50 mM) B supply in *Bacillus boroniphilus* is considerably less compared to other B-tolerant isolates, maintaining much less cellular soluble B even at high B supply. This is proposed to be due to some mechanism of excluding B from the cells of *Bacillus boroniphilus*, by some efflux B transporter like BOR1 that has been reported in *Arabidopsis thaliana* (Takano *et al.*, 2002).

In general, B-tolerant isolates also maintained less pellet-bound B compared to the sensitive strain, *Bacillus subtilis* (Figure 7.1b), but there is no significant correlation between tolerance to B toxicity and pellet-bound B concentration ($p>0.05$). Interestingly, *Bacillus boroniphilus* that cannot grow without B, showed more pellet-bound B at low than at high B supply, in contrast to all other strains. It may be possible that when cells of this unique featured strain exposed to low B supply, B may form some compounds with some membrane-associated proteins or glycolipids but at high B supply, this process may be

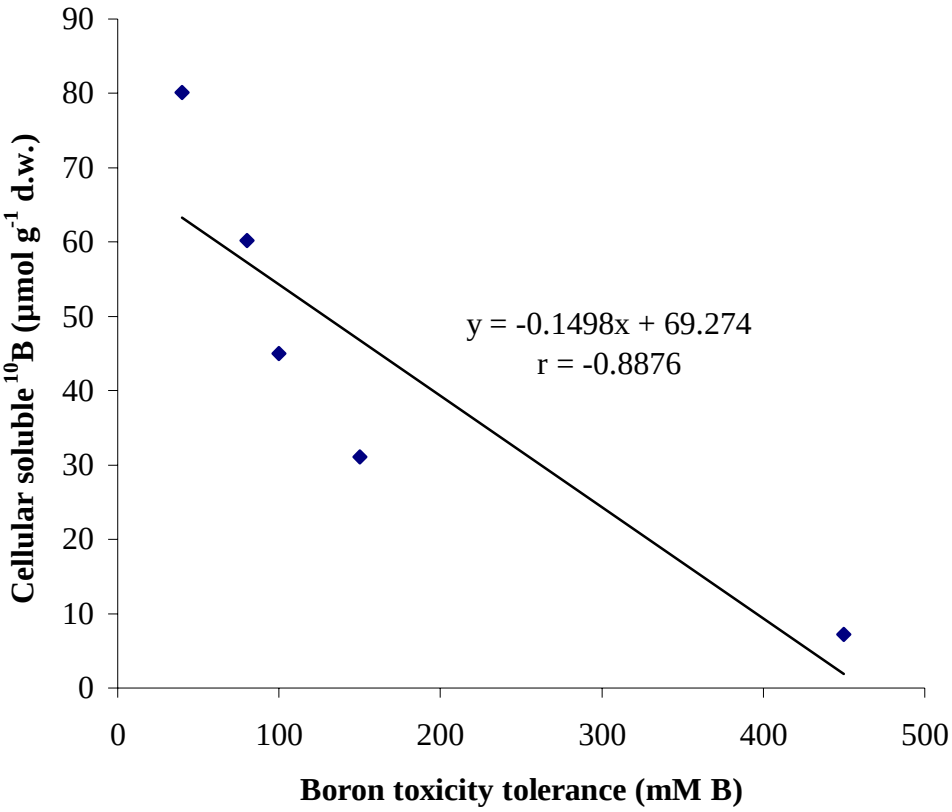


Figure 2. Correlation between boron tolerance and soluble boron in the cell when strains were exposed at 50 mM 10B treatment

inhibited and only form component containing *cis*-diol as is the case with plants (Bolaños, 2004).

The time-course uptake and/or exchange of B between cells and the external medium was determined by measuring soluble ^{10}B and ^{11}B in the cells grown up to mid log phase, first in TSB medium with 5 mM boric acid enriched with ^{11}B and then exposed for a period of 90 min to the same medium with 10 mM boric acid enriched with ^{10}B. Analysis

showed that there was a rapid influx of B across the cell membrane in both B-tolerant and –sensitive species and reached to steady state within a few min. However, the steady-state B concentration in B-tolerant species was 5-fold less than those in B-sensitive species (Figure 7.3), suggesting that an efflux of B is the mechanism by which B-tolerant species were able to maintain a lower B concentration in the cell and thus, in doing so, to prevent the accumulation of toxic cellular B concentration.

Due to initially rapid influx of B to reach the steady-state concentration, the kinetic analysis of B transport across the membrane was not practically feasible. By exposing the cells which were grown first at 5 mM B up to mid log phase, to a concentration of 0.5 mM B, the exchange of B across the membrane was slower than when exposed to high B level (10 mM B), where exchange was more rapid and completely exchanged the ^{11}B with ^{10}B in several min (Figure 7.4). It is predicted that high ^{10}B concentration in the external medium drives this kinetics than ^{11}B concentration in the cell. The difference in rapid establishment of steady-state concentration between both the species suggested the mechanism for exclusion or efflux of B working constitutively. It is also clear from data that after a period of 60 min, there is a decrease in boric acid concentration (Figure 7.3) suggesting that some other mechanism appeared to be activated in *Bacillus boroniphilus* that is able to actively pump boric acid from the cells and thus was able to maintain a lower B concentration in the cell than in the external medium. Hayes & Reid (2004) also provided evidence for active transport of B in relation to B tolerance in barley. Such efflux B transport system against concentration gradient has been reported in *A. thaliana* (Takano *et al.,* 2002) under the control of gene BOR1.

These studies demonstrate that bacterial tolerance to high external B concentration correlates negatively with soluble cellular B. Boron-tolerant strains manages to keep soluble B concentration at lower level in the cell. Based upon this data, it is suggested that there exists some efflux/exclusion mechanism that is operative when B-tolerant strains exposed to

high external B levels. Additionally, carrying a strong efflux B transport system *in Bacillus boroniphilus* that pumps cellular B against concentration gradient (Fig. 7.3, blue line at 90 min) is being suggested.

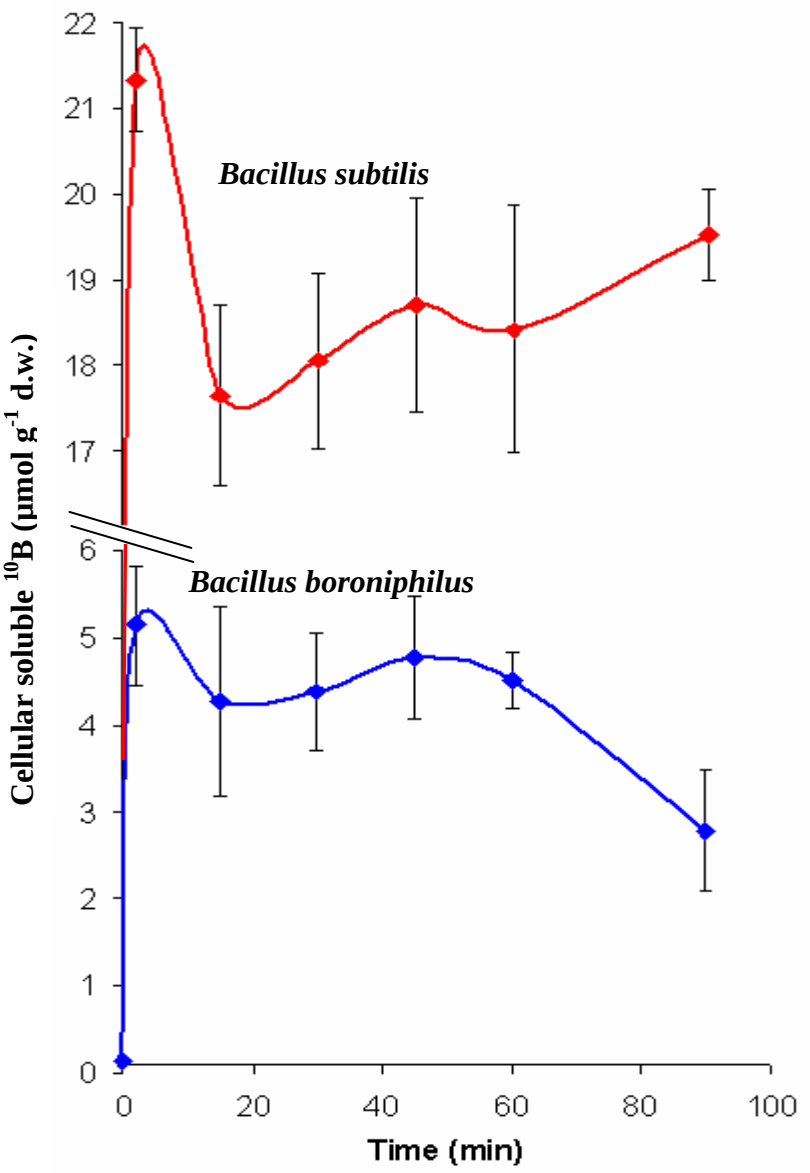


Figure 7.3.

Time-course of B influx and efflux in the cells of *Bacillus boroniphilus* strain T-17s (blue line) and *Bacillus subtilis* (red line) at 10 mM of boron supply. Data are means with error bars (± standard deviation) for four independent replications. d.w., dry weight.

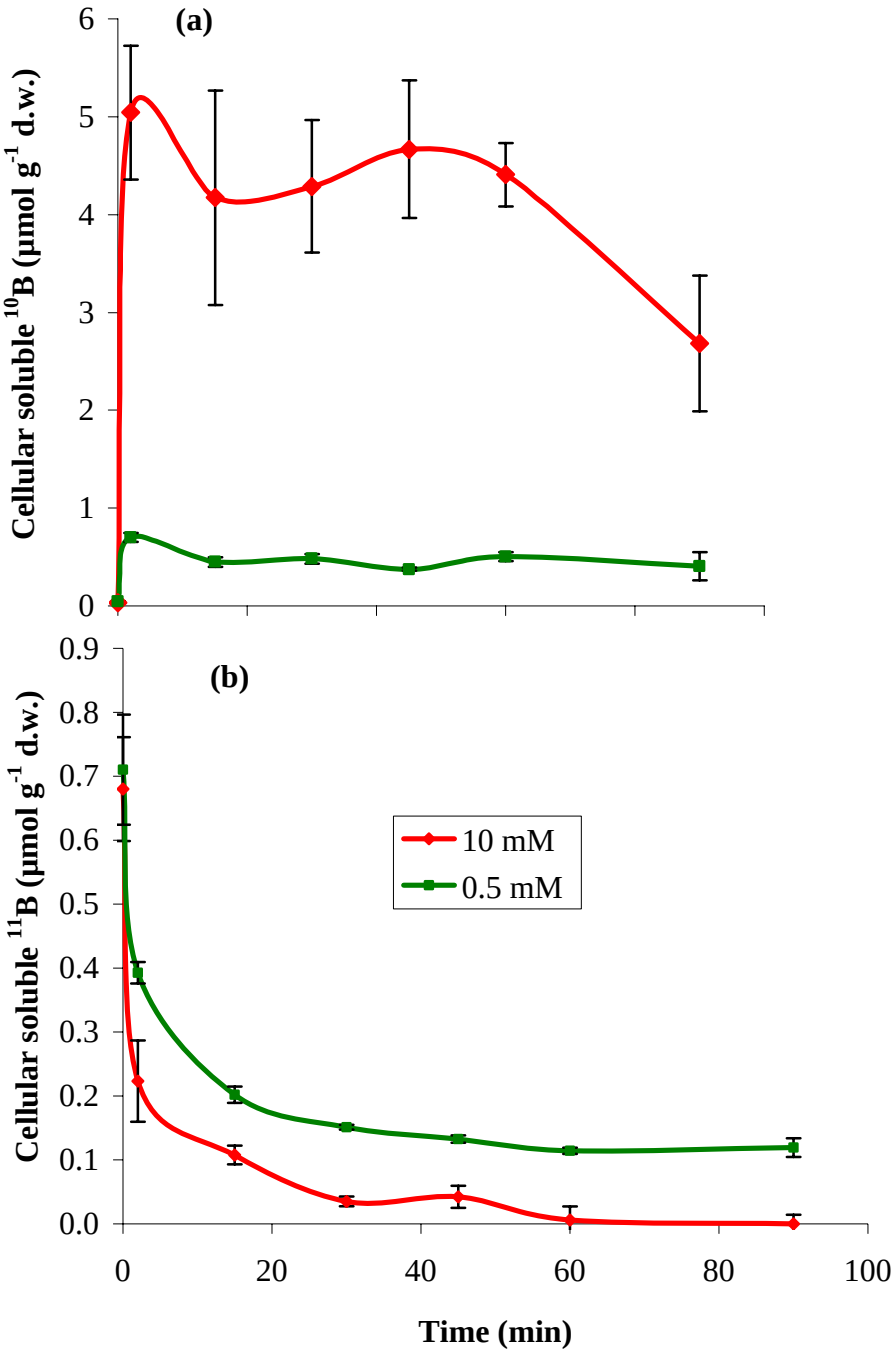


Figure 7.4.

Time-course of B influx and efflux in the cells of strain T-17s at two levels (0.5 and 10 mM) of boron supply. Data are means with error bars (± standard deviation) for four independent replications. d.w., dry weight.

CHAPTER VIII

GENERAL CONCLUSION

Of various extreme environments for the growth of microorganisms, B-toxicity has been presented as a new frontier in extremophiles. It is obvious that we have only scratched the surface in revealing a new vista this regime. Several species of bacteria were examined for this trait and B-tolerant species were identified and characterized in systematic taxonomy. Based upon our taxonomic data, three novel highly B-tolerant species; *Bacillus boroniphilus* sp. nov., *Gracilibacillus boraciitolerans* sp. nov. and *Chimaereicella boritolerans* sp. nov., have been proposed (Ahmed *et al.*, 2007a, b, & c) that were isolated from B-rich soil of Hisarcik area (Kutahya Province, Turkey). These novel species tolerated more than 450 mM B. One B-tolerant strain, *Lysinibacillus boronitolerans* gen. nov. sp. nov., that was isolated from normal soil of greenhouse area (University of Tokyo), has been proposed in a novel genus *Lysinibacillus* gen. nov. (Ahmed *et al.*, 2007d). This novel strain could tolerate 150 mM B and contains lysine – aspartate as diagnostic amino acids in the cell wall peptidoglycans that became the basis for proposal of new genus in addition to other chemotaxonomic data. Based upon our data, the reclassification of two type species as *Lysinibacillus fusiformis* comb. nov. and *Lysinibacillus sphaericus* comb. nov. have also been proposed.

In addition to tolerance against toxic concentrations of B, one novel species could not grow without B, suggesting that B plays an essential role during the growth for some unknown functions. 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) of cell wall; however, RG-II has not yet been identified in bacteria, so the molecular basis for the essentially of B in these novel strains is not clear at the stage.

The time-course uptake and/or exchange of B between the external medium and cells of B-tolerant and/or B–sensitive bacteria showed that there was a rapid influx of B across the cell membrane. However, the steady-state B concentration in B-tolerant species was 5-fold less than those in B-sensitive species (Figure 7.3), suggesting that an efflux of B is the mechanism by which B-tolerant species were able to maintain a lower B concentration in the cell and thus, in doing so, to prevent the accumulation of toxic cellular B concentration.

These studies demonstrate that bacterial tolerance to high external B concentration correlates negatively with soluble cellular B and B-tolerant strains manage to keep soluble B concentration at lower level in the cell. Additionally, the data also showed that after a period of 60 min, there is a decrease in boric acid concentration (Figure 7.3 blue line at 90 min) suggesting that some other mechanism appeared to be activated in *Bacillus boroniphilus* that is able to actively pump boric acid from the cells and thus was able to maintain a lower B concentration in the cell than in the external medium. Such efflux B transport system against concentration gradient has also been reported in *A. thaliana* (Takano *et al.,* 2002) under the control of gene BOR1.

The taxonomic results obtained in this study provide information to the ecological studies for exploration in this regime and thus, provide new topics for further research in the subject of ‘Extremophiles’. 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 and for discussing the biochemical functions of B in living organisms.

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