By screening a bacterial population from the soil in Tokyo, Japan, we isolated a boron-tolerant bacterium, strain BTM4c. Strain BTM4c grew under the boron excess conditions with 100 mM boric acid, which is generally toxic to bacteria. Molecular phylogenetic, chemotaxonomic, and physiological data showed that the strain belongs to the genus \textit{Rhodococcus}, and is to be identified as \textit{Rhodococcus baikonurensis}.

Key words: boron; boron resistance; boron toxicity; \textit{Rhodococcus}

Boron (B) is an essential micronutrient for plants and possibly for animals. In bacteria, heterocystous cyanobacteria have been found to require B for growth and for nitrogen fixation. A biological role of B in quorum sensing has been identified as a structural component of the receptor complex. On the other hand, B is toxic to living cells when present above a certain threshold. To explore B-tolerant microorganism in the natural environment, we have isolated \textit{Algoriphagus bortiotorleans}, \textit{Bacillus boroniphilus}, \textit{Gracilibacillus boracitolerans}, and \textit{Lysinibacillus boronitolerance}. B-tolerate isolates are potential genetic resources for B-resistant genes, which function to adapt an organism to extreme environments. By the use of bioengineering technology, these genes can be used to confer B-tolerance to agricultural crops in naturally B-contaminated areas. Hence, to determine the distribution and characteristics of B-tolerance in bacteria, screening of B-tolerant strains were conducted.

BTM4c was isolated from soil collected in an experimental field of the Yayoi Campus of the University of Tokyo. The soil samples (5 g) were incubated in 50 ml of phosphate-buffered saline (PBS) solution at 30 °C for several d, and boric acid was added incrementally (10 mM B per d). The supernatant was streaked on Luria–Bertani (LB) agar (pH 7.0) plates containing 10 mM B per plate. The strain was subjected to study for further characterization.

Received June 30, 2009; Accepted October 5, 2009; Online Publication, January 7, 2010 [doi:10.1063/1.3119527]
*Rhodococcus* sp. BTM4c, a Boron-Tolerant Bacterium

To demonstrate B-tolerance, strain BTM4c and reference strain *R. baikonurensis* JCM 11411T were grown in tryptic soy broth (TSB, pH 7.2) up to the stationary phase (OD_{600} 1.2) at 30 °C with vigorous shaking. Fresh cells of the strains were dispersed in PBS solution, which was used to inoculate 3 ml of tryptic soy broth (TSB) medium (pH 7.2) with various B levels ranging from 0 (control) to 200 mM B, and were grown with vigorous shaking in test tubes at 30 °C. OD_{600} was measured using a spectrophotometer (TAMPEL, mini Photometer 518R, Tokyo) directly from tubes, and OD_{600} versus time was plotted to obtain growth curves. For comparison of B-tolerance in BTM4c and closely related strains, *R. baikonurensis* JCM 11411T, *R. erythropolis* JCM 3201T, and *R. globulus* JCM 7472T were streaked on TSA containing 100 mM boric acid, and this was incubated for 3d at 25 °C. Among these strains, only BTM4c formed visible colonies (data not shown). Based on this result, it is suggested that the characteristic of B-tolerance is specific to strain BTM4c, but not to *R. baikonurensis* or closely related taxa. This result is in accordance with previous reports that B-tolerance is species/strain specific and has no relationship with closely related phylogenies. *Bacillus boroniphilus* T15zT, which has been isolated as a B-tolerant strain, also requires B for its growth. Hence, the B-requirement for growth was examined. BTM4c grew on TSA without added boric acid (data not shown). To examine the effect of B concentration on cell growth, BTM4c was grown at various levels of boric acid (0, 20, 50, 100, 150, and 200 mM). The type strain of the most closely related species, JCM 11411T, was also analyzed for comparison. BTM4c grew well up to 50 mM B, and still grew in the presence of 100 mM B. In contrast, growth of JCM 11411T was observed up to 20 mM B, but was severely inhibited by 50 mM B (Fig. 1).

The pH of a medium affects bacterial cell growth by itself, but also changes the chemical form of B. Under high-pH conditions, B exists mainly in an anionic form, B(OH)_{4}^{-}, whereas at neutral pH it exists in an uncharged form, which is highly permeable through the lipid bilayer. Hence, cell growth at various pH values was analyzed with B excessive conditions, of 50 mM B. Growth of BTM4c was observed at pH 5.5 and pH 7.2. However, BTM4c did not grow at pH 8.8 in the presence of 50 mM B (Fig. 2). Since BTM4c grew at pH 9 in the absence of B, the anionic form of B must be more toxic to the cells.

For comparison of the effects of borate stress and salt stress on cell growth, strains BTM4c and JCM 11411T were grown with various concentrations of NaCl (0 to 4%). BTM4c grew even in the presence of 4% NaCl in TSA, whereas the growth of JCM 11411T was suppressed severely under these conditions (Fig. 3). This indicates that BTM4c is moderately halotolerant. This is consistent with our previous studies showing that B-tolerant strains such as *Bacillus boroniphilus*, *Gracilibacillus boracitolerans*, and *Lysinibacillus boronitolerans* are moderately halotolerant.3-10

To determine the B concentration in the cells, BTM4c and the reference strains were grown at 25 °C for 2 d in 5 ml of TSB medium containing 100 μM boric acid. Then they were harvested and washed in TSB medium, and...
examined for B content by ICP-MS. The weight of a freeze-dried pellet was also measured to calculate the intracellular B concentration. When the cells were cultured in TSB medium supplemented with 100 \( \mu \)M boric acid, strain BTM4c contained 420 ± 50 nmol g\(^{-1}\) of boric acid in the dried cell pellet, whereas \textit{R. baikonurensis} JCM 11411\(^T\) contained 720 ± 40 nmol g\(^{-1}\). The lower content of intracellular B of BTM4c might contribute the mechanism of tolerance against excessive amounts of B.

Finally, in order to identify other important differentiating traits, we further investigated the physiological and biochemical characteristics of strain BTM4c. During the characterization experiments, all incubations were carried out at 30 \(^\circ\)C unless otherwise stated. The temperature range for optimum growth was determined by up to 2 weeks of incubation at various temperatures on TSA agar medium. The pH range for growth was determined using nutrient broth (NB, Difco) medium adjusted to various pH values (pH 5.0–10.0 at intervals of 1.0 pH unit). The pH was adjusted using KOH or HCl prior to sterilization. Cell morphology, spore formation, and motility were examined under phase-contrast microscopy. Growth under anaerobic conditions was determined after 2 weeks of incubation in an Anaerobic Pack (Mitsubishi Gas, Tokyo). The pH range for growth of BTM4c was 5–10, with optimal growth at 7. No growth was observed at pH 4, and very slight growth was observed at pH 10. The temperature range for growth was 10–37 \(^\circ\)C, with optimal growth at 30 \(^\circ\)C. BTM4c showed tolerance up to 7% NaCl, suggesting that it is moderately halotolerant. No growth of strain BTM4c was observed under anaerobic conditions even after 2 weeks of incubation. Biochemically, strain BTM4c showed positives in both catalase and oxidase activities.

For whole-cell fatty acid analysis, BTM4c was grown on TSA containing 20 mM B at 30 \(^\circ\)C for 24 h, and the cellular fatty acid profile was determined by the GC-based microbial identification system (MIDI) following the manufacturer’s protocols. The genomic DNA of the strain was extracted from cells grown on plates by the method described previously.\(^{15}\) Following digestion with \(P_1\) nuclease, the G + C content of the extracted DNA was determined by HPLC (Shimadzu, Kyoto, Japan; column:Cosmosil 5C18R, Nacalai Tesque, Japan) at a column temperature of 40 \(^\circ\)C and wavelength 270 nm, using the mobile phase as 0.2 mM ammonium phosphate:acetonitrile in a ratio of 40:1. Respiratory quinones were analyzed as described previously.\(^{20}\)

The cellular fatty acid composition of strain BTM4c was as follows: C\(_{14:0}\), 3.9%; C\(_{15:0}\), 2.4%; C\(_{16:1}\)\(\alpha\)c, 1.0%; summed feature 3, 10.3%; C\(_{16:0}\), 30.6%; C\(_{17:0}\)\(\alpha\)c, 1.7%; C\(_{17:0}\), 1.8%; C\(_{17:0}\) 10 methyl, 1.0%; C\(_{18:1}\)\(\alpha\)c, 20.2%; C\(_{18:0}\), 3.2%; tuberculostearic acid (10-methyl-C\(_{18:0}\)), 19.9%; and C\(_{20:0}\), 1.3% (summed feature 3 comprised C\(_{16:1}\)\(\alpha\)c and/or C\(_{15}\) iso 2OH; only components representing more than 1% of the total are reported). This composition is similar to those obtained from \textit{Rhodococcus} species, including \textit{R. baikonurensis}.\(^{21}\) The G + C content of BTM4c was 50.9%, similar to those observed in the type strains of closely related species. The predominant isoprenoid quinone was MK-8, which is consistent with the closely related species of the genus \textit{Rhodococcus}.\(^{22}\)

To determine the distribution and characteristics of B-tolerance in bacteria, we have been exploring B-tolerant strains from the natural environment. So far, strains including \textit{Algoriphagus boritolerans}, \textit{Bacillus boroniphilus}, \textit{Gracilibacillus boracitolerans}, and \textit{Lysinibacillus boronitolerance} have been isolated from the soil. In this study, we obtained a novel member of the B-tolerant bacterial strain, BTM4c, from the soil. Polyphasic taxonomic characterization revealed that strain BTM4c is a member of \textit{Rhodococcus baikonurensis}. This is the first report on the B-tolerance of an actinobacterium within the genus \textit{Rhodococcus}. In view of the chemotaxonomic and molecular phylogenetic data, BTM4c should be a new biotype of \textit{R. baikonurensis}. Under alkaline conditions, the growth of BTM4c was severely inhibited by 50 mM B. This suggests that the anionic form of B was more toxic to the cells. Bor1p plays an important role in yeast tolerance to high levels of B by controlling intracellular boron accumulation.\(^{60}\)

The B permeable channel, AtNIP5;1, and the B transporter, AtBOR1, have been identified in Arabidopsis.\(^{23,24}\) However, to date, the mechanisms that regulate intracellular B-tolerance and transport in bacteria have
not been clearly determined. In this study, several experiments were performed to provide basic microbiological information about the B-tolerant actinobacterial strain BTM4c. Our next molecular genetic analysis of the strain BTM4c, together with other B-tolerant strains isolated previously, should provide new insights into the molecular mechanism of B-tolerance in bacteria.

Acknowledgments

This study was supported in part by a grant from the Japan Science and Technology Agency (JST), Japan.

References