論文の内容の要旨

応用生命工学　　専攻

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氏名　アハマド　イフティカール

指導教員名　　小柳津　広志

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一)

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 (Fig. 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.

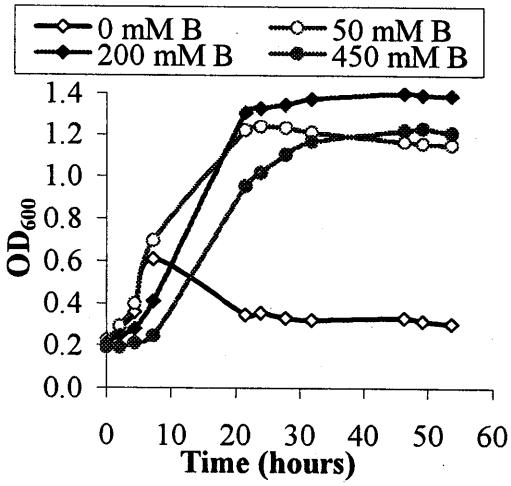

Figure 1. Growth curves of *Bacillus boroniphilus* sp. nov. grown at different levels of B supply in TSB medium.

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.*, 2006a, 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.*, 2006d). 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 (Fig. 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

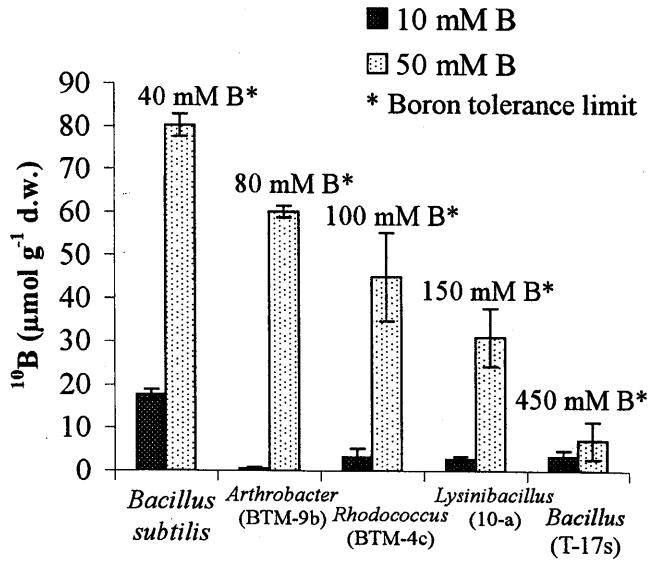

Figure 2. Uptake of boron in cells at two levels (10 and 50 mM) of boron supply for one hour. Data are means with error bars (± SD) for four independent replications. d.w., dry weight.

comparison to sensitive strain i.e. *Bacillus subtilis* as a control (Fig. 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 (Fig. 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 (Fig. 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.

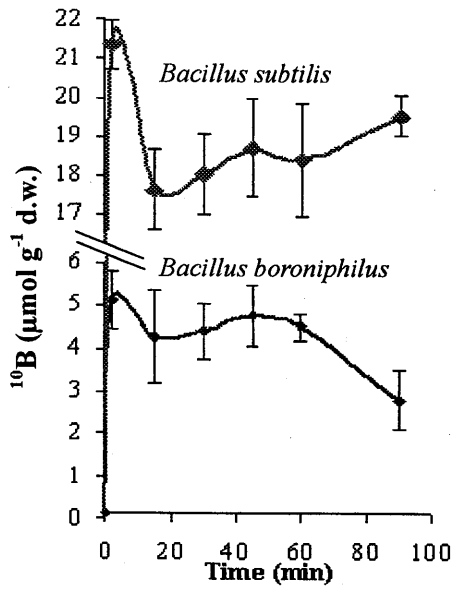

Figure 3. Time-course of B influx and efflux in the cells at 10 mM of boron supply. Data are means with error bars (± SD) for four independent replications. d.w., dry weight.

General conclusions

Bacillus boroniphilus could not grow without B (Fig. 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.

References

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