Turkish Journal of Molecular Biology & Biotechnology
(2017) 2: 21-29

ORIGINAL ARTICLE

Turkish JMBB

Genomic library construction of a boron tolerant bacterium in *Escherichia coli* and selection by boron

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Keywords

Boron, gene, genomic library, tolerance

ABSTRACT • Background and Aims: Boron is a biologically important semi-metal and trace element and essential for some species of the bacteria, animals and plants. Furthermore, the beneficial effects for human are known. Boron is not present in nature by itself. It's in tight relationship with oxygen and exists as boric-acid or sodium, potassium and calcium salts. Boron, while required at low-amounts, is toxic above a certain level for living organisms. Some microorganisms have been found to tolerate higher levels of boron. In this study, we aimed to find the candidate genes that may be involved in boron tolerance in a bacterium isolate belonging to Pseudomonas genus. **Materials and Methods:** In this study, a bacterium isolate belonging to the Pseudomonas genus was used. This bacterium can grow in the tryptic soy broth medium containing 200 mM boric acid. To find candidate genes that may play a role in boron tolerance, genomic DNA of the isolate was extracted to purity and partially fragmented by a restriction enzyme, cloned into a plasmid vector, and transformed into *Escherichia coli. Escherichia coli* is not able grow in boric-acid concentrations above 75-80 mM. Thousands of colonies harboring recombinant plasmids were obtained and then subject to boron selection. The colonies that were found to be tolerant to 110-120 mM boric-acid were selected and DNA sequence analyzes of the inserts in the recombinant plasmids isolated from the clones were performed and the genes within the inserts were identified via bioinformatics approaches. **Results:** Following boric-acid selection of the colonies containing the genomic library of the isolate, a total of 5 insert regions of the recombinant plasmids were sequenced. The genes identified include the activities for cyanate hydratase, major facilitator superfamily permease protein, RND family efflux transport protein, membrane fusion protein subunit and the carbomyl-phosphate synthase major subunit. **Conclusion:** This study provides candidate gene information regarding boron tolerance in bacteria. Among the activities, we suggest that the proteins of major facilitator superfamily permease and membrane fusion protein are particularly promising and interesting considering the insert sizes and potential functions.

INTRODUCTION

Boron (B) is an essential micronutrient required by vascular plants (1) and present in the structure of plant cell wall (2). Although most of the studies have been conducted to elucidate the role of B in plants, there is some relatively limited number of studies concerning other living organisms. It was

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shown that B is also needed or utilized by some animals (3, 4) and single celled eukaryotes (5). Some species of bacteria such as *Bacillus boroniphilus* (6) and certain Cyanobacteria species (7) were reported to require boron as an essential element for their growth.

Other noteworthy information regarding B includes that the cyanobacterium *Nostoc spongiaeforme var. tenue* and *Streptomyces griseus* produce boron- containing antibiotics (8). Boron was also found to promote nitrogen fixation in some Azotobacter species (9) and plays a role in quorum sensing in *Vibrio harveyi* (10). Phenyl boric acid catabolism was described in *Arthrobacter nicotinovarans* (11), where B is mostly eliminated as ortho-boric acid [$B(OH)_3$]. All the findings taken together suggest strongly that boron plays diverse and important biological roles in the living organisms.

To understand the relationship between boron and biological life from the molecular perspective, it is important to find and use suitable model organisms in order to decipher the biomolecules employed, including specific genes, proteins, metabolites and so on. Thus, this study aimed at proposing some candidate genes involved in boron tolerance in one of the model bacteria species. A genomic library of a highly boron tolerant bacterium was constructed in *Escherichia coli (E. coli)* and subject to boron selection by incubating the recombinant colonies in the media containing much higher levels of boric acid than the wild type strain could survive. Isolation and sequencing of the inserts of the recombinant plasmids yielded gene information that can potentially provide boron tolerance in bacteria.

MATERIALS and METHODS

Bacterial isolate, genomic DNA isolation and 16SrRNA gene sequence analysis

In this study, a bacterium strain that was isolated from Kütahya-Hisarcık boron mine was used (BÇ, unpublished results). The isolate named as KH17b was obtained purely and saved as a glycerol stock at -80°C. The isolate was grown on tryptic soy broth agar medium containing 50 mM boric acid using classical microbiological methods. The cell pellet was prepared by scraping the cells and resuspended in 500 ml sterile phosphate buffer saline (PBS) solution. Genomic DNA (gDNA) was isolated from the cell pellet using the phenol-chloroform method as described before (12). gDNA quality and amount was assessed by absorbance measurements at 260, 230 and 280 nM and by agarose gel electrophoresis analysis.

The 16S rRNA gene region was amplified with 27F and 1492R universal primers as described before (13,14) and polymerase chain reaction (PCR) product was sequenced (Macrogen, Netherlands). Bioinformatics analyzes were conducted using Bioedit (15), Mega softwares (16) and NCBI-Blast service *(https://blast.ncbi.nlm.nih.gov/Blast.cgi).*

Plasmid isolation and growth conditions for genomic library studies

A number of vectors were used for genomic library construction studies but as a result of the optimizations, pACYCDuet1 was chosen as the vector. pACYCDuet1 (Novagen) is a plasmid of around 4 kb in length with a chloramphenicol (Cm) antibiotic resistance cassette. Cm concentration used for the selection of the plasmid was 25 µg/ml in Luria Bertani (LB) medium. DH10b cells were grown at 37°C in LB liquid or solid media containing the antibiotic and boric acid, when needed. Sterile boric acid stock solution was prepared at 0.5 M at pH 7 and the dilutions were made to the desired final concentrations, as needed. The plasmid isolations were conducted using the plasmid miniprep kit as described by the manufacturer (Qiagen, Germany). The plasmids were transformed to *Escherichia coli* DH10b cells using the heat shock method (12).

Genomic library construction

About 10 µg genomic DNA of the KH17b isolate was partially digested with *MboI* restriction

enzyme (New England Biolabs, USA). To obtain the fragment sizes ranging from 500 bp to 5 kb, enzyme amount and reaction time were optimized for each genomic DNA preparation. Three microgram of the plasmid pACYCDuet1 was cut with *BamH*I and dephosphorlyated using shrimp alkaline phosphatase (Fermentas). The digested plasmid was then purified from the gel using a plasmid miniprep kit (Fermentas) following agarose gel electrophoresis analysis. The fragments of digested gDNA and plasmid DNA were combined in a tube and were purified using a column- based PCR purification kit (Qiagen, Germany). First, second and third elutions were obtained in a volume of around 20 µl each. For ligation, T4 DNA ligase with its buffer (Fermentas) was added to the mixture at the suggested amounts and the reaction was completed to a final volume of 20 µl and the mixture was first incubated at room temperature for one hour followed by incubation at 16°C overnight in a thermocycler (Eppendorf). Next day, 2-3 µl from the ligation mixture was used to transform into *E. coli* DH10b cells using the heat shock method (12). The cells were incubated overnight at 37°C on LB agar plates containing Cm. If the colonies were observed and cloning was successful, the remaining ligations mixtures were transformed the next day and thousands of colonies were obtained as "genomic library". To assess whether the plasmids in the colonies carry inserts or not, colony-PCR was conducted on randomly selected colonies. If the insert sizes are satisfactory, *E. coli* colonies containing the genomic library of the isolate were then subject to boron selection.

Selection with boron

E. coli DH10b colonies harboring the recombinant plasmids which contain the genomic fragments of the isolate in the insert regions were subject to boron selection by plating on LB-cm plates with various levels of boron. First, the cells were plated on LB-cm plates containing 80 mM boric acid. Then, every colony that grew over night at 37°C was picked by a sterile toothpick and streaked on the LB-cm plates containing 90 and 100 mM boric acid. After incubation for 1-3 days at 37 °C, the colonies that were able to grow were picked and subject to boron tolerance spot tests as explained below.

Boron tolerance spot test

First, the cell mass for the test was obtained by growing the pure colonies on the LB-cm plates not containing boric acid. Next day, the cells of the colony were scraped from the plates and resuspended in 250 µl sterile PBS. The intensity of the suspension was determined by measuring the absorbance values at 600 nm using a visible spectrophotometer (Optizen, South Korea). The final concentration of the suspension was adjusted by sterile PBS to be OD 0.5, which is the value to be used as the first dilution indicated as the 1/1 dilution having the final volume of 500 µl. Then, serial dilutions were performed to obtain 1/2, 1/4, 1/8, and 1/16 dilutions in a final volume of 500 µl each for every clone that was tested. Also, *E. coli* DH10b cells containing the plasmid with no insert was used as the negative control [DH10b (pACYCDuet1)]. Five µl from each dilution of the corresponding clone was taken and spotted on the LB-cm plates containing 0, 80, 90, 100, 110 and 120 mM boric acid. The growth of the clones were assessed and the pictures of the plates were taken after 1-5 days of incubation. Growth and no growth were indicated by stars and dashes, respectively, for all of the clones and the negative control to interpret the results.

Determination of plasmid insert sequences and some bioinformatics analyzes

After the clones tolerant to boron were selected following boron tolerance tests, the plasmids were isolated from the bacteria using commercial plasmid mini-preparation kits (Qiagen and Fermentas). The control for the presence and size of the inserts were carried out by polymerase chain reaction

using the primers ACYCDuetUp1 (GGATCTCGACGCTCTCCCT) and ACYCDuetDown1 (GGATTATGCGGCCGTGTACAA). The inserts of the recombinant plasmids were sequenced (Macrogen, The Netherlands) using the same primers. The sequences were analyzed by using BlastX and BlastN (NCBI, *http://blast.ncbi.nlm.nih.gov/blast.cgi*) and the program Bioedit (15) to find out about the activities potentially encoded by the nucleotide sequences cloned in the inserts of the recombinant plasmids.

RESULTS

In this study, a bacterium isolate that can survive in the tryptic soy medium (TSB) containing 200 mM boric acid was used. This was found to be closely related to a Pseudomonas species based on 16SrRNA gene sequence similarity (Çöl B., unpublished results).

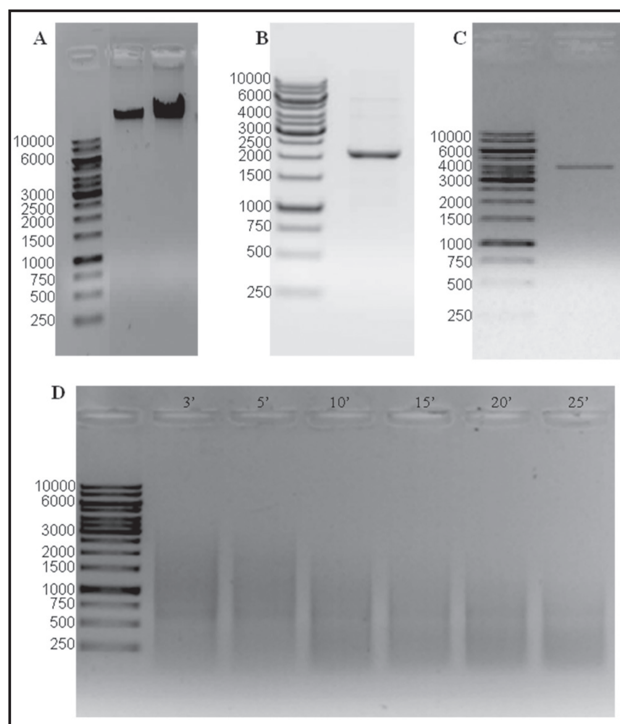

Figure 1 The representative results of the standard experiments used in genomic library construction. A, agarose gel image of the genomic DNA of the boron tolerant bacterium; B and C, the plasmid pACYCDuet1 purification results; D, agarose gel image of the genomic DNA fragments created by *Mbo*I partial digestion. First lanes of the cells were loaded with a DNA size marker, the numbers next to marker bands indicate the sizes in base pair and the minute numbers on gel D represents the digestion times.

For genomic library construction, DNA isolation from the isolate was carried out using the phenol-chloroform extraction (12) (Figure 1A). The plasmid pACYCDuet1 was purified and prepared at a desired concentration for cloning (Figure 1B and 1C). The conditions for the partial digestion of the genomic DNA by the restriction enzyme *Mbo*I (Fermentas) were optimized to obtain the desired fragments sizes and 5 µl of the 1/100th dilution of the enzyme was incubated with approximately 10 µg genomic DNA for a duration of 2 minutes (Figure 1D).

After ligation of the pure gDNA fragments with the digested and dephosphorylated pure plasmid, transformation into *E. coli* DH10b produced 5.000 colonies after incubation at 37 °C for one day (Figure 2). All of the colonies were scraped from the plates and made into glycerol stocks to be saved at -80 °C as the “genomic library” of the boron tolerant bacterium.

Boron selection

The transformant colonies were first subject to pre-selection on the LB-cm plates containing 80 mM boric acid. As a result of this selection, a number of 180 colonies were observed on the plates. These colonies were then selected one by one and streaked onto the LB-cm plates with no boric acid. After the colonies were grown again, fresh cells were streaked by toothpicks on the LB-cm plates containing 90 and 100 mM boric acid. At the end of incubation, a number of thirty colonies that exhibited growth were selected and the levels of boron tolerance of these colonies were determined by boron tolerance spot test. Different dilutions of the colonies prepared as described in Materials and Methods section were spotted to be 5 µl each on the LB-cm plates containing 0, 80, 90, 100, 110 and 120 mM boric acid. After incubation at 37 °C for 1-4 days, the growths of the clones were visually

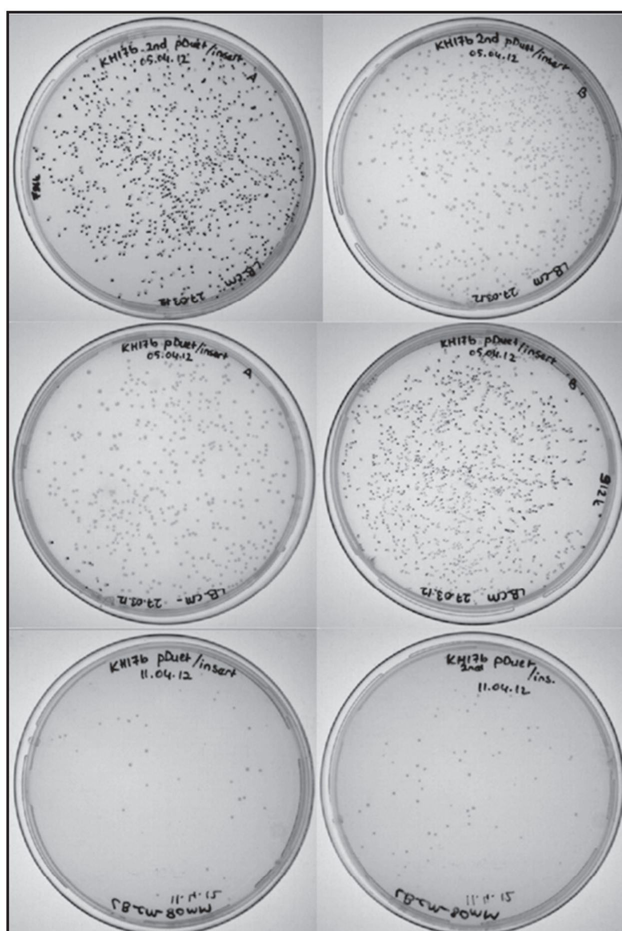

Figure 2 **Representative plate pictures of *E. coli* DH10b cells containing the recombinant plasmids of the genomic library.** The colonies were obtained by incubating the *E. coli* cells after transformation of the ligations mixtures which possessed gDNA fragments of the boron tolerant bacterium and the corresponding digested plasmids. The colonies forming the genomic library were counted to be around 5000.

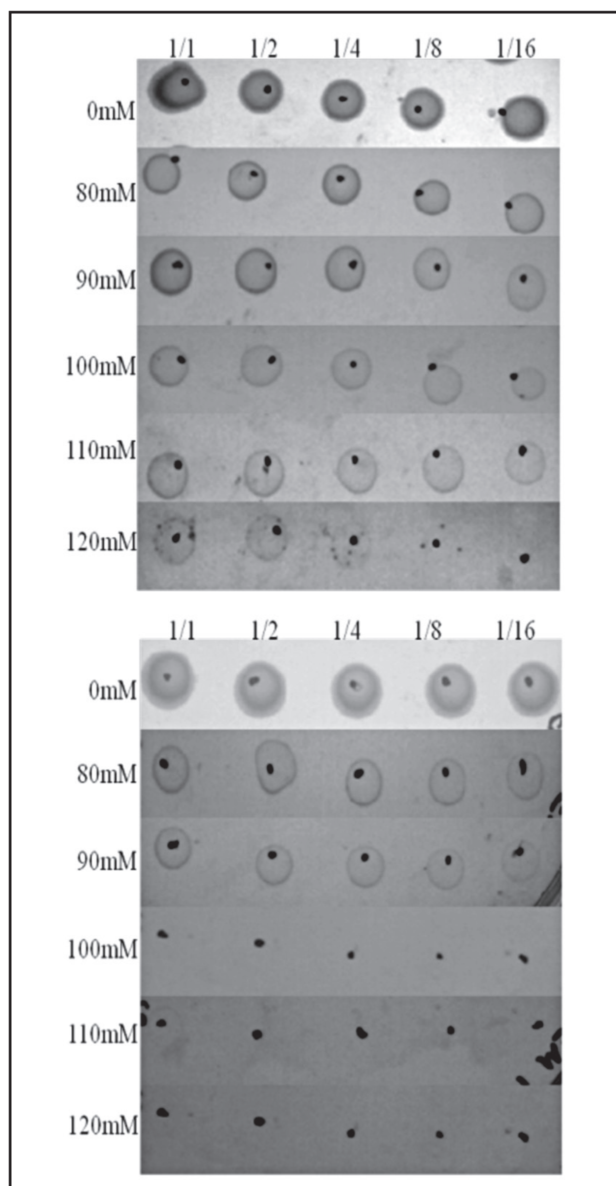

Figure 3 **Boron tolerance spot test results of a tolerant clone obtained after boron selection and the negative control DH10b (pACYCDuet1).** The figure was created by the pictures of the results of boron tolerance spot tests. Each clone was grown on a number of six individual plates containing 0, 80, 90, 100, 110 and 120 mM boric acid, where the dilutions of the cells used were 1/1 (OD0,5), 1/2, 1/4, 1/8, and 1/16 as indicated by numbers on the figure. The picture above was created by cutting and pasting the images of the growth results obtained from the individual plates in order to comparatively show the growth levels. The picture on the bottom is of the negative control, while the one the top shows the growth of the clone containing a recombinant plasmid on various levels of boron (boric acid levels in mM are shown on the figure).

inspected and the photographs were taken. An example of the picture is given in Figure 3. The levels of the growth were determined by eye observing the intensity of the spots and comparing with that of the negative control [DH10b (pACYCDuet1: no insert)]. The results portraying the growth levels of the clones from each dilution were expressed as “stars” and summarized in Table 1. As a result of these experiments, the clones p2a9, p2c43, p2d17, p2d23 and p2d33 were shown to tolerate higher levels of boron as compared to the negative

control and other clones (Figure 3 and Table 1). Thus, these clones were made into the glycerol stocks and taken to further analysis, where the recombinant plasmids were isolated.

Table 1 Boron tolerance spot test results of the tolerant colonies obtained after boron selection

	0 mM	80 mM	90 mM	100 mM	110 mM	120 mM
p1a2	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	✶✶⅄⅄⅄	⅄⅄---	⅄----
p1a3	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	✶✶⅄⅄⅄	⅄⅄---	⅄⅄---
p1a5	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	✶✶⅄⅄⅄	⅄⅄---	⅄----
p1a7	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄⅄---	⅄----
p1a12	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄⅄---	⅄----
p2a3	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄---	⅄----
p2a8	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄---	⅄----
p2a9	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	✶⅄⅄⅄⅄
p2a10	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	✶✶✶⅄⅄	⅄----	⅄----
p2a11	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	✶✶✶⅄-	⅄----	⅄----
p2a12	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄--	⅄----	⅄----
p2a13	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄--	⅄----	-----
p2a14	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄---	⅄----	⅄----
p2a15	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄----	-----
p2a16	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄---	⅄----	-----
p2b47	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄⅄---	⅄----
p2b48	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄⅄---	⅄----
p2c41	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄⅄---	⅄----
p2c43	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄⅄⅄⅄⅄	⅄⅄⅄⅄⅄
p2d18	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄⅄---	⅄----
p2d17	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄⅄⅄⅄⅄	⅄⅄⅄--
p2d19	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄-	⅄⅄---	⅄----
p2d20	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄-	⅄⅄---	⅄----
p2d21	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄-	⅄----	⅄----
p2d22	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄--	⅄----	-----
p2d23	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄⅄⅄⅄⅄	⅄⅄⅄⅄⅄
p2d24	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄⅄---	⅄----
p2d25	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄--	⅄⅄---	⅄----
p2d26	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄--	⅄----	-----
p2d27	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄--	⅄----	-----
p2d30	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄⅄⅄--	⅄⅄---
p2d33	✶✶✶✶✶	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	⅄⅄---	⅄⅄---
DH10b(pDuet)	✶✶✶✶✶	✶✶✶✶✶	⅄⅄⅄⅄⅄	-----	-----	-----

The symbols (✶), (⅄) and (-) indicate strong, weak and no growth, respectively. 5 spots from left to right represent the dilutions of 1/1, 1/2, 1/4, 1/8 and 1/16.

Isolation of plasmids and insert analyzes

In order to isolate the recombinant plasmids in the clones, the clones were grown by spreading single colonies on LB-cm plates and incubating at 37°C for 2 days. The cells mass was obtained by scraping the cells from the plates and then resuspending and washing in sterile PBS solution. Then, the plasmids were isolated from the cells using commercial kits (Qiagen or Fermentas). The inserts in the plasmids were sequenced by Sanger sequencing method (Macrogen, The Netherlands) using the primers given in the previous section. Finally, Blast analyzes at NCBI suggested the following activities present in the inserts: cyanate hydratase, major facilitator superfamily permease, membrane fusion protein and carbamoyl-phosphate synthase large subunit. The details of the results including the insert sizes are given in Table 2.

DISCUSSION

Boron is an important trace element required by the plants and other organisms at lower amounts (1). However, depending on the organism type, this element exerts toxic effects at certain concentrations (3, 4). Due to this characteristic of boron, some agricultural fields cannot be used and higher levels of boron decrease crop yield and cause economical loss (17). Some organisms, on the other hand, can tolerate toxic levels of boron. The molecular mechanisms employed in achieving the phenotype of boron tolerance either directly or indirectly can be investigated by finding and proposing candidate genes and proteins by using the tolerant organisms as the model organisms. In this study, we have used a bacterial isolate (KH7b) that can grow in the presence of 200 mM boric acid as the model organism to get clues about boron tolerance in Pseudomonas genus.

There are a number of ways to search for the genes relating to boron tolerance. One of the approaches is to construct the genomic library of the organism that has the phenotype of tolerance and check to find out if the specific DNA regions from the organism can provide *E. coli* with relatively more tolerance. In this study, such efforts resulted in proposing some gene activities that may be considered as candidates that could be involved in boron tolerance in bacteria.

As a result of several selections and controls, a number of 5 recombinant plasmids, which have the genomic fragments of the KH16b isolate, were shown likely to provide *E. coli* DH10b strain with higher boron tolerance value of 110-120 mM boric acid.

E. coli DH10b p2a9 was found to tolerate 120 mM boric acid, whereas the negative control could tolerate 80-90 mM boric acid. The DNA region cloned in the insert region of this plasmid has a very high sequence similarity to the gene encoding the part of the enzyme cyanate hydratase.

Cyanate hydratase is an enzyme included in EC 4.2.1.104 group and hydrolyses cyanate (*http://www.genome.jp/dbget-bin/www_bget?ec:4.2.1.104*).

Table 2 Blast analysis results of the insert sequences of the recombinant plasmids

Plasmid name	Approximate insert size (bp)	Boric acid tolerance level	BlastX analysis of the insert sequence (protein or gene name / percent similarity)
p2a9	100	120 mM	Cyanate hydratase %100
p2c43	500	110 mM	Major facilitator superfamily permease %96
p2d17	200	110 mM	Membrane fusion protein %87
p2d23	200	110 mM	Membrane fusion protein %87
p2d33	250	120 mM	Carbamoyl-phosphate synthase large subunit %97

Some bacteria may tolerate cyanate toxicity through this enzyme (18). It has also been stated in the literature that this gene may also be involved in providing tolerance to salt stress (19). Since boric acid (borate) is also a kind of salt, a relationship between this enzyme activity and boron tolerance is not far from consideration. However, it should be noted here that the insert size within the plasmid is relatively small. The protein cyanate hydratase contains 149 amino acid residues and the region that was cloned in p2a9 and matches with the full enzyme falls within the region from 88 through 108 amino acid residues within the C terminal domain (cyanate lyase domain). This particular domain is responsible for the hydrolysis of cyanate. It is presently early to propose a correlation between this activity and boron tolerance and more experimental evidence is certainly needed, but a homolog of cyanate hydratase is present in *Pseudomonas stutzeri*. Furthermore, when the amino acid sequence of Pseudomonas cyanate hydratase was analyzed by BlastP to search for similarities in *E. coli*, it was found that CynS protein (20) shows 39% identity and 67% "positive" residues to the Pseudomonas enzyme. We propose that one of the future experiments could involve the comparison of boron tolerance of Δ*cnyS* mutant of *E. coli* with the wild type.

E. coli DH10b strain containing the p2c43 plasmid was found to tolerate 110-120 mM boric acid in LB-cm medium. The insert within the plasmid has a size of around 500 bp and shows high sequence similarity (96%) to the major facilitator superfamily permease protein (MFS transporter) based on Blast analyzes. The MFS transport super family group is one of the two major membrane transport protein families found in living organisms. The other group is ABC transport proteins. The membrane proteins in the MFS group can pump a variety of molecules into and out of the cell functioning as a solute uniport, solute/cation simport, solute/cation antiport and/or solute/solute antiport. Among the molecules carried across by the MFS permease membrane proteins include simple sugars, oligosaccharides, inositols, drugs, amino acids, nucleosides, organophosphate esters, metabolites of the Krebs cycle and many types and organic and inorganic anions and cations (21).

The studies with *Saccharomyces cerevisiae* identified a membrane protein responsible for boron tolerance. This protein, ATR1, belongs to the MFS transport protein family. When ATR1 is overexpressed in a plasmid in yeast, it was found to provide the cells with both relatively higher boron and salt tolerance (22). Therefore, this specific activity of the MFS transport protein family found in this study is likely significant and should be considered as a candidate gene for bacterial boron tolerance.

p2d17 and p2d23, two different plasmids, were found to possess relatively small inserts (~200 bp) and the level of boron tolerance for the colonies containing these plasmids was around 110-120 mM boric acid. Blast analysis of the insert sequences revealed an 87% similarity to a membrane fusion protein (MFP). After scrutinization of the matching sequences, it appears that the insert region may potentially encode a RND family efflux protein, a part of MFP subunit (TIGR01730). It may be speculated that this activity may be involved in providing boron tolerance through membrane association or interaction.

Boron tolerance of *E. coli* strain carrying the plasmid p2d33 with an insert size of about 250 bp is around 100-120 mM boric acid. Analysis of the insert DNA sequence resulted in gene information similar to the carbomyl-phosphate synthase major subunit, albeit a relatively short match of 32 amino acid residue. Repeated boron tolerance spot test experiments showed that this clone exhibited less tolerance than the others, that the growth in 100 mM boric acid was visible but the growth on the plates containing 110 mM and 120 mM boric acid was really weak, where only the spots inoculated with the cells of 1/1 and 1/2 dilutions were shown to contain faint density. There is also no link found

between this activity and any stress phenotype in the literature. Therefore, for these reasons, this activity should not be a priority to be considered as a candidate gene in boron tolerance.

Acknowledgements: *This study is supported by TÜBİTAK (The Scientific and Technological Research Council of Turkey) (project number 112T614), Muğla SK University BAP division (project number 12/104).*

REFERENCES

1. Warington K. The Effect of Boric Acid and Borax on the Broad Bean and certain other Plants. Annals of Botany 1923;37:629-672.
2. O'Neill MA, Ishii T, Albersheim P, Darvill AG. Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. Annu Rev Plant Biol 2004;55:109-139.
3. Rowe RI, Bouzan C, Nabili S, Eckhert CD. The response of trout and zebrafish embryos to low and high boron concentrations is U-shaped. Biological Trace Element Research 1998;66:261-270.
4. Rowe RI, Eckhert CD. Boron is required for zebrafish embryogenesis. Journal of Experimental Biology 1999;202:1649-1654.
5. Lewin JC. Boron as a growth requirement for diatoms. Journal of Phycology 1966;2:160-163.
6. Ahmed I, Yokota A, Fujiwara T. A novel highly boron tolerant bacterium, Bacillus boroniphilus sp. nov., isolated from soil, that requires boron for its growth. Extremophiles 2007;11:217-224.
7. Mateo P, Bonilla I, Fernandez-Valiente E, Sanchez-Maeso E. Essentiality of Boron for Dinitrogen Fixation in Anabaena sp. PCC 7119. Plant Physiol 1986;81:430-433.
8. Dembitsky VM, Smoum R, Al-Quntar, et al. Natural occurrence of boron-containing compounds in plants, algae and microorganisms. Plant Science 2002;163:931-942.
9. Anderson GR, Jordon JV. Boron: A Non-Essential Growth Factor for Azotobacter Chroococcum. Soil Science 1961; 92:113-116.
10. Chen X, Schauder S, Potier N, et al. Structural identification of a bacterial quorum-sensing signal containing boron. Nature 2002;415:545-549.
11. Negrete-Raymond AC, Weder B, Wackett LP. Catabolism of arylboronic acids by Arthrobacter nicotinovorans strain PBA. Appl Environ Microbiol 2003;69:4263-4267.
12. Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual. 2001.
13. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 1991;173 (2): 697–703.
14. Jiang H, Dong H, Zhang G, Yu B, Chapman LR, Fields MW. Microbial diversity in water and sediment of Lake Chaka, an athalassohaline lake in northwestern China. Applied and Environmental Microbiology 2006;72 (6): 3832–45.
15. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 1999;41:95-98.
16. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0. Molecular Biology and Evolution 2016;msw054.
17. Nable RO, Bañuelos GS, Paull JG. Boron toxicity. Plant and Soil 1997;193:181-198
18. Sung YC, Fuchs JA. Characterization of the Cyn Operon in Escherichia-Coli-K12. Journal of Biological Chemistry 1988;263:14769-14775.
19. Qian D, Jiang L, Lu L, Wei CH, Li Y. Biochemical and Structural Properties of Cyanases from Arabidopsis thaliana and Oryza sativa. Plos One 2016;6.
20. Anderson PM, Sung YC, Fuchs JA. The cyanase operon and cyanate metabolism. FEMS Microbiol Rev 1990;7:247-252.
21. Pao SS, Paulsen IT, Saier MH Jr. Major facilitator superfamily. Microbiol Mol Biol Rev 1998;62:1-34.
22. Kaya A, Karakaya HC, Fomenko DE, Gladyshev VN, Koc A. Identification of a Novel System for Boron Transport: Atr1 Is a Main Boron Exporter in Yeast. Molecular and Cellular Biology 2009;29:3665-3674.