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ORIGINAL PAPER

In vivo evolutionary engineering of a boron-resistant bacterium: *Bacillus boroniphilus*

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Abstract Boron is an industrially and biologically important element. However, the mechanisms of boron tolerance and its transport in bacteria and many other living systems are still not clearly understood. In this study, the boron resistance level of a boron-tolerant bacterium, *Bacillus boroniphilus* DSM 17376, was improved up to 300 mmol l^{-1} boron, by employing an in vivo evolutionary engineering strategy based on batch selection under continuous exposure to gradually increasing boron stress levels. The resistance was heterogeneous within the final mutant population which ranged from about 1- to 16-fold of the wild type resistance at 150 mmol l^{-1} boron stress level. Boron-resistant mutants had significant cross-resistance to iron and copper stresses, and were also cross-resistant to salt (NaCl) stress, suggesting a common resistance mechanism between these stress types. Additionally, highly boron-resistant mutants had up to 2.8-fold higher boron contents than the wild-type, when exposed to high levels of (150 mmol l^{-1}) continuous boron stress throughout their cultivation. It was shown that evolutionary engineering is a successful approach to significantly increase bacterial boron resistance and investigate the complex mechanism of boron tolerance and transport in microbial systems.

Keywords *Bacillus boroniphilus* · Boron resistance · Evolutionary engineering · Stress resistance

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Introduction

Boron is an industrially important element of semiconductor group, having properties between metals and non-metals (Bolanos et al. 2004). It occurs in nature as borates in sedimentary rocks, oceans, coal and some soils (Howe 1998). The predominant supplies for boron come from California and Turkey, where tectonic activity also helps supply a mechanism for the introduction of boron in the environment (Argust 1998). It has a wide range of industrial applications in manufacture of glass and other vitreous products, perborates, agriculture, wood preservation, fire retardancy, and more recently, in the pulp and paper and ceramics industries (Schubert 2003). Additionally, boron dopants are used in semiconductor industries (Kazanskii et al. 2002) and in

electrochemical technologies in wastewater treatment (Chen 2004). Recently, it was also shown that organoboron compounds can be used in tin-free methods to run radical reactions necessary in drug discovery projects and other industrial processes (Darmency and Renaud 2006).

Boron is also a biologically important element. In most biological fluids with almost neutral pH values, boron is mainly (about 96%) found as boric acid, $B(OH)_3$, and a small amount of borate anion $B(OH)_4^-$ (Bolanos et al. 2004). Boron is an essential micronutrient for all vascular plants (Warington 1923), and is also nutritionally important for some unicellular eukaryotes such as diatoms (Lewin 1966) and yeasts (Bennett et al. 1999); animals and human (Rowe et al. 1998; Rowe and Eckhert 1999; Nielsen 2002). Boron is essential for heterocystous cyanobacteria (Bonilla et al. 1990) and actinomycetes of the genus *Frankia* (Bolanos et al. 2002). These specific bacteria require boron for their envelope stability. Under N_2-fixation conditions, the envelopes prevent diffusion of oxygen and thus protect the enzyme nitrogenase from inhibition by oxygen (Bonilla et al. 1990; Bolanos et al. 2004). The stabilization of the envelopes by boron is rather complex and depends on the chemical composition of the envelopes. In the heterocysts, boron is found in an inner laminated layer that consists of specific glycolipids. In *Frankia*, boron is located in multilaminate vesicle walls. These vesicle walls consist of glycolipids and neutral lipids including long-chain polyhydroxy fatty acids or alcohols. Their abundant free diol groups enable bonding with borate (Bolanos et al. 2004). A boron-containing quorum-sensing signal molecule has also been identified in bacteria (Chen et al. 2002). In plants, boron is required for physical strength of cell walls and for cell adhesion (Miwa and Fujiwara 2010). However, high concentrations of boron are toxic for plants and other organisms. Boron exposure has been discussed as a potential cause of chronic kidney disease in Southeast Asia (Pahl et al. 2005). Adverse effects of boron on male reproduction in laboratory animals has also been reported, however, no clear evidence of male reproduction effects of boron was obtained from human studies involving highly exposed workers (Scialli et al. 2010). Boron toxicity has also been discussed in insects, considering the use of boron compounds for preserving lumber from insect attack and decay by bacteria and fungi, and to treat cockroach and flea infestations (Gentz and Grace 2006).

Although recent studies provided insight into the mechanism of boron transport in plants by revealing transporters responsible for efficient boron uptake and for tolerating high boron levels (Miwa and Fujiwara 2010), the mechanisms of boron transport and tolerance is still not clearly understood in other organisms, including bacteria.

Recently, a gram-positive, motile, rod-shaped and highly boron-tolerant bacterium was isolated from boron-containing soil of Hisarcik area in Kutahya, Turkey. Based on phenotypic, chemotaxonomic and phylogenetic analyses, it was shown that the bacterium belonged to a novel *Bacillus* species and was named as *B. boroniphilus* (Ahmed et al. 2007a). In a more recent study, a variety of boron-tolerant soil bacteria, including *B. boroniphilus* as the most-tolerant species among all, were tested for their boron uptake upon pulse exposure to high and low levels of boron in the growth medium. Statistical analysis of the data showed that the more tolerant the species, the less protoplasmic boron content it had. Thus, a high boron efflux and/or exclusion were suggested as a boron tolerance mechanism in the bacteria tested (Ahmed and Fujiwara 2010).

Evolutionary engineering makes use of evolutionary principles based on random mutation and selection to improve industrially important microbial properties (Sauer 2001). Spontaneous or induced mutagenesis drives the take-over of the culture by fitter variants periodically, under continuous cultivation conditions (Dykhuizen and Hartl 1983). Successful applications of this methodology on different industrially important microorganisms are found in the literature, including evolutionary engineering of cobalt resistance (Çakar et al. 2009), accelerated utilization of glucose, xylose, and arabinose (Wisselink et al. 2009), xylose fermentation (van Maris et al. 2007; Sonderegger and Sauer 2003), L-arabinose fermentation (Wisselink et al. 2007; Wiedemann and Boles 2008), lactose consumption (Guimaraes et al. 2008), and multiple stress resistance (Çakar et al. 2005) in the baker's yeast *Saccharomyces cerevisiae*. Other examples include evolutionary engineering applications with industrially important bacteria, such as improvement of plasmid stability in *B. subtilis* by selective chemostat cultures, chemostat selection of *Escherichia coli* mutants with improved physiological

properties (Weikert et al. 1997), improvement of acetate resistance of *Acetobacter aceti* (Steiner and Sauer 2003), and selection of quiescent *E. coli* with high metabolic activity (Sonderegger et al. 2005).

In this study, an in vivo evolutionary engineering strategy was applied for the first time to a boron-tolerant bacterium *B. boroniphilus* to further improve its boron-tolerance level and investigate the mechanism of high boron resistance in this bacterium. A highly boron-resistant mutant population was obtained by batch selection under continuous stress conditions with gradually increasing boron levels. A high level of heterogeneity was observed in boron-resistance of single mutant cells obtained from the final mutant population. The boron contents and cross-resistances of boron-resistant mutants to other stress types were also determined to have an insight into boron resistance mechanism.

Materials and methods

Strain, media and growth conditions

Bacillus boroniphilus (DSM No: 17376) was purchased from DSMZ (the German Resource Centre for Biological Material, Germany) and used as the initial wild-type strain in this study. Unless otherwise stated, TSB (DSM Medium 220 + 2.5% NaCl + 0.05 mol l^{-1} H_3BO_3 + 10 μg ml^{-1} $MnSO_4$) medium (pH 7.4 ± 0.1) was used for cultivations. DSM Medium 220 contained 15 g pancreatic casein digest, 5 g soy peptone and 5 g NaCl per liter. Cultivations were performed in 50 ml-culture tubes under aerobic conditions at 30°C and 150 rpm. Cell growth was monitored by measuring optical density at 600 nm (OD_{600}). From all selection cultures, aliquots were taken along the exponential growth phase, and stored at −80°C in TSB medium containing 35% (v v^{-1}) glycerol.

Ethyl methane sulfonate (EMS, Sigma) mutagenesis was performed (Lawrence 1991) to result in 10% of survival rate after the chemical treatment. Briefly, 0.5 ml overnight culture with an OD_{600} about 0.5 was washed twice with an equal volume of 50 mmol l^{-1} KH_2PO_4-buffer (pH 7). This solution was then exposed to 500 μl EMS and incubated at 30°C for 60 min in a screw-cap glass tube. At the end of the mutagenesis procedure, freshly prepared 500-μl sodium thiosulphate solution (10%, w v^{-1}) was added to the tube for neutralization. The solution was mixed well and cells were collected upon centrifugation at 14,000 rpm for 10 min. The supernatant was discarded and the cell pellet was washed twice with Medium 220 and incubated later in liquid TSB medium for overnight growth of the surviving population at 30°C. This overnight culture was used as the starting population for all selection experiments in this study. It was also used in screening experiments to determine the boron concentration which reduces growth by 50%.

Evolutionary selection strategies to increase boron resistance

The evolutionary selection strategy employed in this study was based on selection under continuous exposure to boron stress, where the stress levels were gradually increased upon each successive batch cultivation (Fig. 1). Briefly, EMS-mutagenized initial *B. boroniphilus* population was inoculated into a 50 ml-culture tube containing 10 ml TSB medium with 55 mmol l^{-1} H_3BO_3. Following 24 h of cultivation, the culture was centrifuged at 13,000 rpm for 5 min in a benchtop centrifuge and washed twice with fresh TSB medium. It was then inoculated into the same H_3BO_3-containing TSB medium at an initial OD_{600} of 0.03. The procedure was repeated for 50 times, where the H_3BO_3 concentration in the medium was increased at every passage in the fresh medium until no growth was observed. This corresponded to a final H_3BO_3 concentration of 300 mmol l^{-1} in the final population. For each selection step, a culture aliquot was withdrawn, centrifuged at 14,000 rpm for 5 min, washed twice with fresh medium and stored at −80°C in 35% (v v^{-1}) glycerol. The standard TSB medium with 50 mmol l^{-1} H_3BO_3 concentration was used as the negative control for boron stress selections.

Estimation of stress resistance

Stress resistance against a variety of stress conditions was determined by using a high-throughput, most-probable number (MPN) assay, as well as by spotting assays, i.e. serial dilutions on solid culture media. The MPN assay is used to determine viable cell numbers (Russek and Colwell 1983), and is a reliable method in estimation of stress resistance (Çakar et al. 2005; Çakar et al. 2009). It is based on serial dilutions

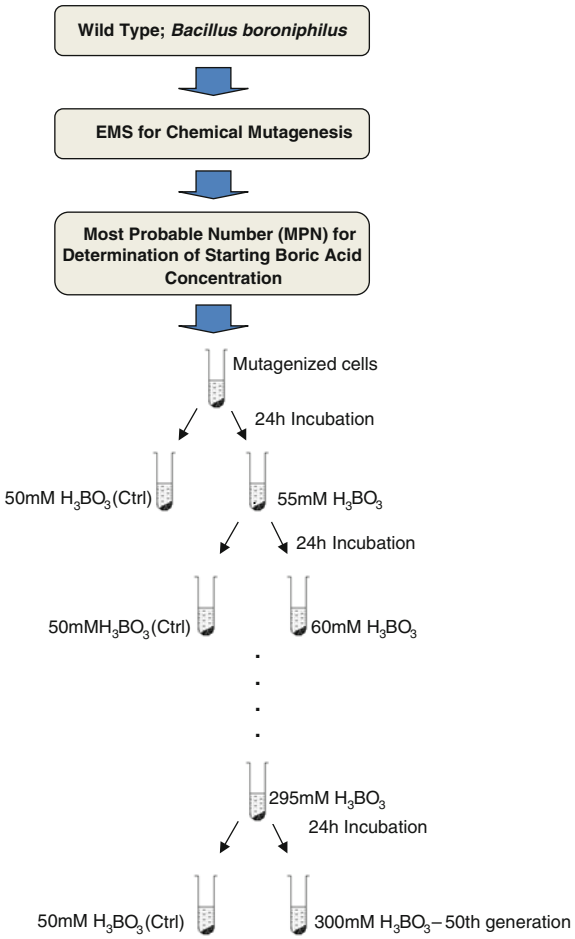

Fig. 1 Experimental selection protocol under continuous boron stress conditions. Following EMS mutagenesis, the cell population was exposed to continuously applied boron stress for 50 passages, where boron concentration was gradually increased at each transfer by 5 mmol l^{-1}, between 55 and 300 mmol l^{-1}

made in the range of 10^{-1} to 10^{-8} for five parallel samples in 96-well plates containing 180 µl TSB. Based on the ability of cells to grow in higher dilutions, the most probable number of survivors was determined by using standard MPN tables (Lindquist 2010).

Resistance to boron stress was determined by cell growth in 96-well plates for 48 h using TSB medium with 150 mmol l^{-1} H_3BO_3 and estimating the viable cell numbers and survival rate by MPN assay.

Resistance to other stress conditions including iron, zinc, copper, cobalt, chromium, and salt (NaCl) stress were determined first by spotting assay on solid TSB media containing 0.5–2 mmol l^{-1} $FeCl_2$, $ZnCl_2$, $CuCl_2$, $CoCl_2$, $CrCl_3$, and 5% (w v^{-1}) NaCl. Resistance to iron, copper, salt stress as well as sorbitol, ethanol, oxidative (H_2O_2), heat and freeze–thaw stress conditions were determined also by MPN assay, using 0.5–2 mmol l^{-1} $FeCl_2$, $CuCl_2$, 5% (w v^{-1}) NaCl, 5% (v v^{-1}) sorbitol, 5% (v v^{-1}) ethanol, 55°C heat and −20°C freeze–thaw stress conditions, and by incubating plates for 48 h. For heat shock stress, 1 ml liquid culture of each mutant was exposed to 55°C for 10 min in a thermomixer, and then cooled on ice. Freeze–thaw stress was performed by exposing 1 ml of each mutant culture to −20°C for 2 h. After this process, cells were thawed, harvested by centrifugation at 14,000 rpm for 5 min and washed twice with TSB medium. MPN method was then applied to harvested cells. Stress resistance was expressed as 'survival rate', and calculated by dividing the number of stress-treated and viable cells to that of non-treated cells (Çakar et al. 2009).

Principal component analysis (PCA)

Principal component analysis (PCA) was used to compare the stress resistance behavior of individual resistant mutants selected and to compare different stress responses. For this purpose, a PCA MATLAB code was used.

Determination of boron contents

Cells were inoculated into 1 l-Erlenmeyer flasks containing 200 ml TSB medium with 150 mmol l^{-1} boric acid. The initial OD_{600} value of the cultures was 0.04. They were cultivated for 72 h at 30°C. Pellets were obtained by centrifugation at 5,000 rpm for 15 min. The pellets were then washed twice with distilled water, dried at 70°C for 24 h, cooled down in a desiccator for 30 min and cell dry weights were determined. For hydrolysis of cell pellets, 5 ml 10 mmol l^{-1} HNO_3 was used, and the solution was incubated at 105°C for 2 h. Boron contents of both initial medium and culture supernatants were determined by flame atomic absorption spectrophotometer (FAAS). A Varian AA 280 FS flame atomic absorption spectrophotometer (Varian AA 280 FS, Australia) equipped with an acetylene-N_2O burner was used. A coded boron hollow cathode lamp was used as the spectral radiation source. The wavelength and

spectral slit width were set to 249.8 and 0.2 nm, respectively. Results for each sample were given as the means of three repetitive measurements.

Boron contents per cell dry weight were calculated by using the following equation:

$$\text{Boron held by cells} = \frac{(\text{B content of initial medium} - \text{B content of supernatant}) \times \text{Medium vol.}}{\text{Cell dry weight}}$$

Results and discussion

Screening for determination of initial boron stress level

Prior to selection experiments, a *B. boroniphilus* culture was EMS-mutagenized to obtain a genetically diverse initial population for selection experiments. For both wild-type and EMS-mutagenized *B. boroniphilus* named as EMS4 here, minimum inhibitory concentrations for H_3BO_3 were determined. For this purpose, they were cultivated in TSB media containing H_3BO_3 in a range between 50 and 1000 mmol l^{-1}. Results showed that the survival rates of both cultures dropped below 0.001 at H_3BO_3 levels of 300 mmol l^{-1} and higher. About 100 mmol l^{-1} H_3BO_3 reduced the cell density by 50% of both the wild-type and EMS4 culture (Fig. 2). EMS4 showed slightly higher survival rates compared to wild type strain. Thus, the EMS-treatment did not seem to increase the sensitivity of the cells to boron ions in the population. By considering these results, 55 mM boric acid stress was chosen as the initial stress level for in vivo selection.

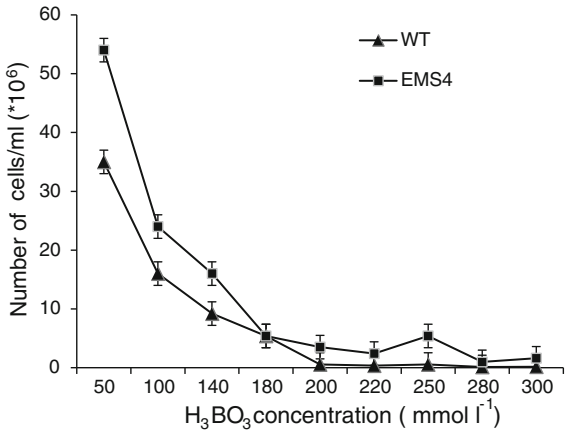

Fig. 2 The boron concentration for 50% inhibition of growth of wild-type and EMS mutagenized *B. boroniphilus*. *Triangles* indicate number of viable cells per ml of the wild-type, and *squares* indicate number of viable cells per ml of the EMS-mutagenized culture. The results were obtained via MPN test, where the initial OD_{600} values for each culture were the same

Selection of boron-resistant populations and characterization of boron resistance at the single cell level

In vivo evolutionary engineering under continuous exposure to gradually increasing boron stress levels was employed as the selection strategy throughout this study (Fig. 1). Due to the boron requirement for the growth of this bacterium, as indicated in the literature (Ahmed et al. 2007a), cultures grown in standard TSB medium containing 50 mmol l^{-1} boric acid were used as control groups. Boron stress levels were increased gradually at each successive batch culture by 5 mmol l^{-1}. Thus, by starting at 55 mmol l^{-1} initial H_3BO_3 stress level, 50 mutant populations were obtained, and the final mutant population survived 300 mmol l^{-1} H_3BO_3, beyond which no growth occurred (Fig. 1). Ahmed et al. (2007a) reported the isolation of three strains of *B. boroniphilus* (T-14A, T-15Z^T and T-17 s), and in that study, the growth curve of strain T-17 s revealed that it survived 450 mmol l^{-1} boron. They reported that the three strains had similar phenotypic and phylogenetic characteristics. As the strain T-15Z^T had the highest similarity with *B. jeotgali* (AF 221061) and some other *Bacillus* species, T-15Z^T was deposited in culture collections, under the number DSM 17376 (Ahmed et al. 2007a), which is the strain used in our study. Our screening results with the DSM 17376 strain showed that, in our cultivation conditions, about 100 mmol l^{-1} H_3BO_3 reduced the cell density by 50% of the initial culture, and no survival beyond 300 mmol l^{-1} was observed.

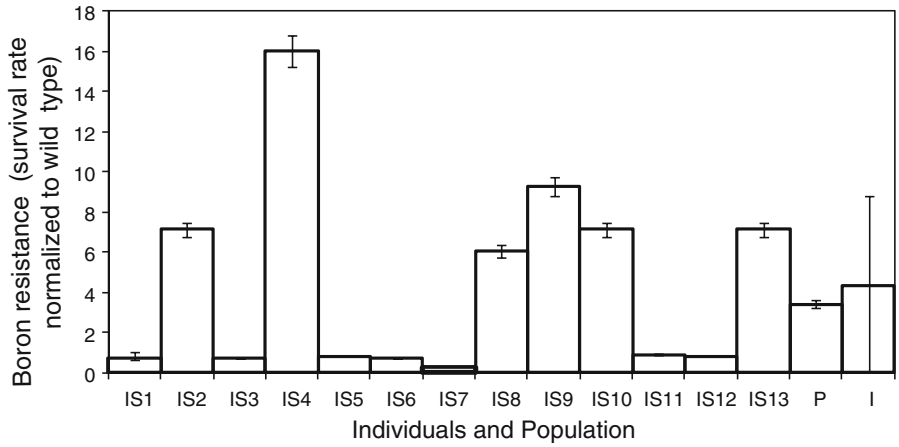

Fig. 3 Determination of survival rate of *B. boroniphilus* population and individuals isolated from continuous, increasing H_3BO_3 stress selection strategy at 150 mM H_3BO_3. IS1–IS13 are selected individual mutant clones of final evolved population. *P* represents the final population and *I* indicates the arithmetic mean resistance value for the 13 individual mutants tested, respectively. The survival rates of each individual and wild-type cells were determined by the MPN upon incubation at 30°C for 48 h in the presence of 150 mM H_3BO_3. The boron resistance was then expressed as the fold increase in survival rate normalized to that of wild-type. The results are the mean values of five completely independent experiments, and the error bars indicate standard deviations

However, using evolutionary engineering approach, boron resistance of the initial culture was significantly improved up to 300 mmol l^{-1}.

The final mutant population was spread on solid TSB medium containing 20 mmol l^{-1} boric acid, and after 48 h of incubation at 30°C, 13 individual colonies were picked randomly and named as (*IS1, IS2,…. IS13*). To determine the boron resistance levels of individual mutants; selected individuals, the final mutant population and the wild type strain were tested for their resistance against 150 mmol l^{-1} H_3BO_3, using MPN assay. According to the MPN assay results, *IS2, IS4, IS9, IS10* and *IS13* showed significantly higher boron resistance than the wild type strain and were chosen for further characterization. Successive batch cultivations in the absence of boron showed that the selected boron-resistant mutants were genetically stable (data not shown). Among all mutants tested, *IS4* showed the highest boron resistance which was up to about 16-fold of the wild-type level. Significant variation was observed among the stress resistance levels of mutant individuals, which indicated a heterogeneous mutant population (Fig. 3). Such a heterogeneity was also observed in our previous studies regarding evolutionary engineering of multiple-stress resistant (Çakar et al. 2005) and cobalt-resistant (Çakar et al. 2009) yeast, which suggests that the boron resistance mechanism in *B. boroniphilus* is also complex and possibly involves several genes.

Estimation of cross-stress resistance of boron-resistant mutants to other stresses

The selected five highly boron-resistant mutants were further characterized by estimation of their potential cross-resistance to a variety of other stresses such as iron, zinc, copper, cobalt, chromium, salt (NaCl), osmotic (sorbitol), ethanol, oxidative (hydrogen peroxide), heat and freeze–thaw stress. The cross-resistance tests were performed first by spotting assay on solid media and by MPN assay in 96-well plates. The spotting assay results revealed that the mutants were not cross-resistant to zinc, chromium and cobalt stress (data not shown). However, significant cross-resistance to iron, copper and salt stress were observed, particularly in the case of the mutant individual *IS4* (Fig. 4).

To determine the cross-resistance levels of the mutants quantitatively, MPN assay was applied. The results showed that most of the mutants were more resistant to iron ($FeCl_2$) stress than the wild-type. Particularly, the survival rates of *IS4* and *IS9* were about 5 and 2, respectively, which indicates that these two mutants could grow even better in the presence of 1 mmol l^{-1} $FeCl_2$ in the medium than in the

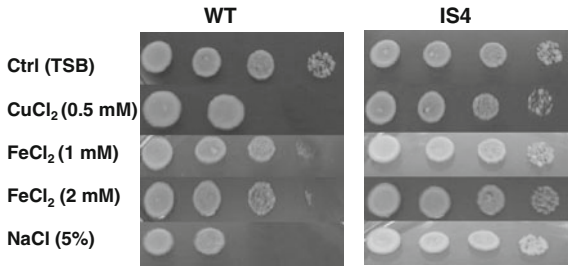

Fig. 4 Cross-resistance solid plate test results of wild-type and boron-evolved mutants to $CuCl_2$, $FeCl_2$ and NaCl. The results are based on serial dilution (from *left* to *right*: 10^{-1} to 10^{-4}) and growth on solid TSB plates at 30°C for 72 h in the presence of stress factors

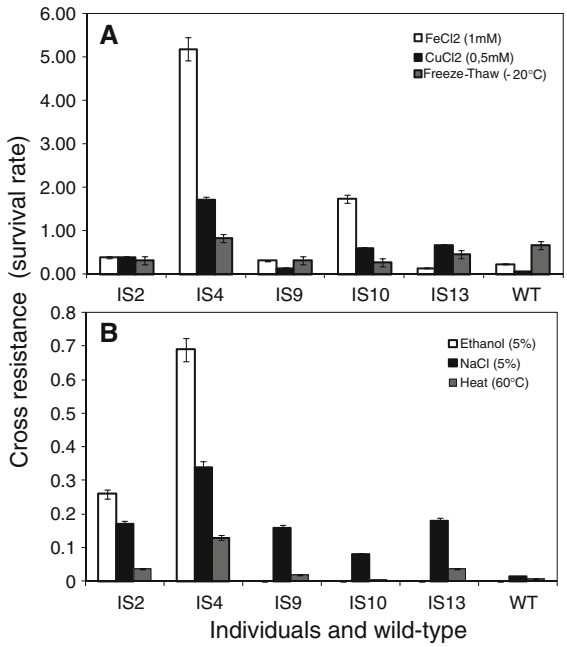

Fig. 5 **a** Cross-resistances of selected mutant individuals and the wild type to $FeCl_2$ (1 mM), $CuCl_2$ (0.5 mM) and freeze–thaw (−20°C) stress, as determined by the MPN method. **b** Cross-resistances of mutant individuals to ethanol (5%, v v^{-1}), NaCl (5%, w v^{-1}), and heat (60°C) stresses (please note that the scale on *y*-axes of Fig. 5**a**, **b** are different, indicating a higher level of cross-resistance against $FeCl_2$, $CuCl_2$ and freeze–thaw stresses. The results are the mean values of five completely independent experiments, and the error bars indicate standard deviations

control medium (Fig. 5a). The survival rate of *IS4* was about 23-fold of that of the wild-type. Similarly, all mutants were more resistant to $CuCl_2$ stress. *IS4*, with a survival rate of about 2, could grow better in the presence of 0.5 mmol l^{-1} $CuCl_2$ in the medium than in the control medium (Fig. 5a). Its survival rate was about 24-fold of that of the wild-type. Regarding freeze–thaw stress (−20°C), among all mutants tested, only *IS4* showed a slightly higher cross-resistance than the wild-type (Fig. 5a).

Although the survival rate values were low, all mutants tested had a significantly higher salt (5%, w v^{-1} NaCl) stress resistance than the wild-type. *IS4* had again the highest resistance level under salt stress conditions, with a survival rate of about 23-fold of the wild-type (Fig. 5b). In plants, there are some recent studies on the combined effects of salinity and boron stresses on plants which commonly occur in natural systems (Yermiyahu et al. 2007; Yermiyahu et al. 2008; Martinez-Ballesta et al. 2008; Smith et al. 2010; Banuelos et al. 2010; Grieve et al. 2010). Different boron-tolerant bacteria isolated from soil were usually found to tolerate between 3 and 11% (w v^{-1}) NaCl (Ahmed et al. 2007b, c; Miwa et al. 2009). These results may suggest a possible common mechanism evolved between salt and boron tolerance and/or transport. Regarding ethanol (5% v v^{-1}) stress, only *IS4* and *IS2* had significantly higher resistance levels than the wild-type (Fig. 5b). Similarly, most of the mutants tested had significantly higher resistance to heat stress (60°C) than the wild type, *IS4* again being the most heat-resistant strain with a survival rate of about 19-fold of the wild type (Fig. 5b). MPN analyses revealed no significant cross-resistance of the mutants to sorbitol (5% v v^{-1}) and oxidative (1 mmol l^{-1} H_2O_2) stresses (data not shown). The results revealed that the most boron-resistant mutant *IS4* did not only gain high resistance to boron stress, but also to a variety of stresses such as iron, copper, ethanol, salt and heat stress (Fig. 5a, b).

Principal Component Analysis (PCA) was applied to the cross-resistance data to clarify the differences between the responses of mutant individuals to various stresses, and also to understand which stress condition(s) caused differences in response. PCA results revealed that the most boron-resistant mutant *IS4* showed a significantly different response to diverse stress types than the wild type (Fig. 6a). Indeed, it showed a significantly high level of cross-resistance against a variety of stresses, compared to the other boron-resistant mutants and the wild-type. PCA analysis also revealed that the response variance was high between mutant individuals and the wild-type, regarding $FeCl_2$ and $CuCl_2$ stresses (Fig. 6b).

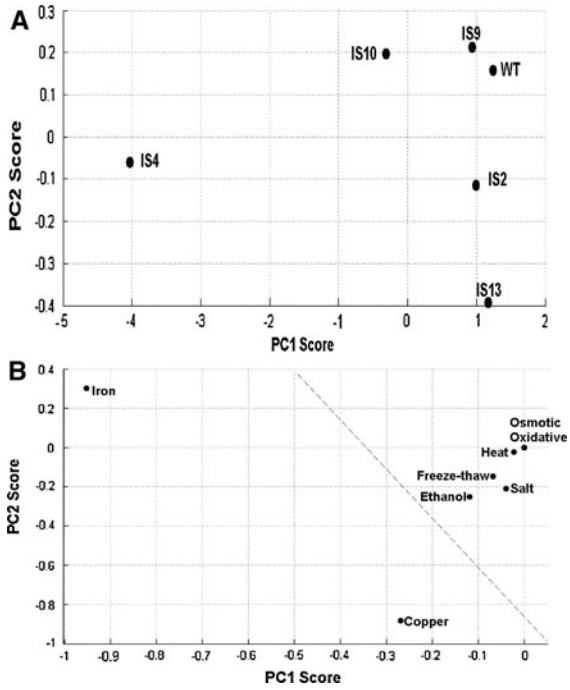

Fig. 6 **a** Principle component analysis (PCA) of mutant individuals and the wild-type with respect to their cross-resistance test results. **b** PCA of the effects of different stress types on mutant individuals and the wild-type strain

As the majority of the boron-resistant mutants were cross-resistant to iron and copper stresses, and as the mutant *IS4* with the highest boron resistance showed highest tolerance to both iron and copper stresses, it can be concluded that iron and copper tolerance/uptake might be related with boron tolerance/uptake metabolism. Regarding PCA, the eigenvalue analysis of covariance matrix indicated that the ratio of two largest eigenvalues to the rest of eigenvalues was 99%, indicating a good representation of the data. Toxic concentrations of Mn, Al, B, Na, Cl, and Fe are commonly found on agricultural soils (White and Brown 2010). However, to our knowledge, there are no reports in the literature on any cross-resistance studies involving boron and iron. Similarly, no relationship between boron and copper tolerance could be found, either. There is only one report on potassium effects on plants, relating boron and copper contents. It was shown that application of potassium decreased plant boron levels and use of potassium fertilizers lowered copper content of alfalfa forage (Daliparthy et al. 1994). Our cross-resistance data regarding iron and copper indicate that there is likely to be a common tolerance/transport mechanism between boron, iron, and copper.

Determination of boron contents of boron-resistant mutants

To determine the amount of boron associated with the cells, selected mutant individuals with high boron resistance and the wild type strain were cultivated for 72 h in batch cultures in the presence of 150 mmol l^{-1} boron in the medium. Boron contents were determined by FAAS. The results showed that the cellular boron content of the mutant with the highest boron resistance, IS4, was about 3.5-fold of that of the wild-type. Additionally, the other boron-resistant mutants also had higher boron contents than the wild type, ranging between 1.2 to about 2.8-fold of the wild-type boron content (Fig. 7). In plants, boron efflux has been reported as the major boron-tolerance mechanism (Hayes and Reid 2004). Similar results were obtained with yeast, when mild levels of boron stress were applied as a pulse during early exponential phase of growth (Kaya et al. 2009). However, the boron-resistance level of *B. boroniphilus* is significantly higher than those organisms. Thus, to investigate bacterial boron-tolerance mechanism, Ahmed and Fujiwara (2010) determined boron contents of a variety of boron-tolerant soil bacteria exposed to boron, the most tolerant of which was *B. boroniphilus* strain T-17 s. *Bacillus subtilis* strain ISW 1214 was used as a control in that study. Cells were exposed to

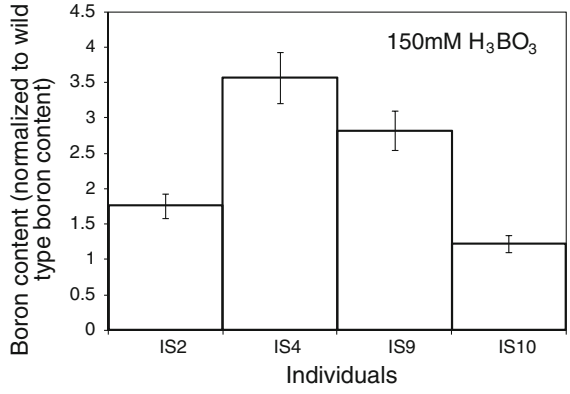

Fig. 7 Boron contents of mutant individuals determined by Flame Atomic Absorption Spectrometry (AAS). Cells were grown for 72 h at 150 mM H_3BO_3 stress level, prior to AAS measurements. The data are reported as mean values of three experiments

high (50 mmol l^{-1}) and low (10 mmol l^{-1}) concentrations of boric acid as a pulse stress for 1 h, after which the boron contents were determined. Statistical analysis of the results indicated that the lower the protoplasmic boron concentration of a bacterial species, the higher its boron resistance level. Under the experimental conditions tested, high boron efflux/exclusion was suggested as the bacterial boron-tolerance mechanism. In our study, however, the boron-tolerance level of the *B. boroniphilus* strain was improved and both the initial wild-type and the improved boron-resistant mutant strain were exposed to a significantly higher (150 mmol l^{-1}) boron concentration throughout the whole cultivation (72 h). Our results revealed that, at high boron concentrations, the mutant *B. boroniphilus* strain with the highest boron resistance (*IS4*) had also higher boron content than the wild type. It should be noted that, in our study, a comparison of boron resistance and boron contents within the same species was made for the first time, whereas the study by Ahmed and Fujiwara (2010) is based on a comparison between different bacterial species. Additionally, the resistance mechanism at significantly higher levels of boron and under continuous exposure throughout the whole cultivation might be different than the mechanism under pulse boron stress conditions applied at lower levels. Our results suggest that boron-resistance mechanism in *B. boroniphilus* is rather complex and should be investigated in more detail, including genetic studies.

Conclusions

In this study, an in vivo evolutionary engineering strategy based on batch selection at gradually increasing boron levels was used to improve boron-resistance level of *B. boroniphilus.* Highly boron-resistant mutants were obtained which could resist up to 300 mmol l^{-1} boron in the medium. However, boron resistance of single mutant cells within the evolved population was highly heterogeneous, implying a complex boron resistance mechanism involving multiple genes. Significantly high cross-resistance levels of boron-resistant mutants to iron and copper were observed which may suggest a common resistance mechanism in *B. boroniphilus* between boron and these metals. Similarly, cross-resistance of boron-resistant mutants to salt stress should also be considered for further investigations. Finally, our mutants with improved boron-resistance levels had higher boron contents than the wild type cells, when exposed to high levels of boron (150 mmol l^{-1}) throughout the cultivation. These results suggest that in *B. boroniphilus*, continuous exposure to high levels of boron might trigger a different resistance mechanism than pulse exposure to low boron levels which seem to activate boron efflux and/or exclusion mechanism. Further detailed investigations including genetic studies are necessary to clarify the molecular mechanism of boron resistance in *B. boroniphilus*.

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Conflict of interest The authors declare that they have no conflict of interest.

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