ABSTRACT

Boron (B), the fifth element is of pivotal biological importance in maintaining viable plant growth without causing mortal harm to biotic community of the surrounding soil. Over use of boron based compounds might result in causing boron toxicity and alteration of tolerance of boron by tolerant bacteria, capable of growing in presence of boron in atmosphere up to a certain level. In the present study, boron tolerant bacterial strains were isolated from soil samples with prolonged and chronic overexposure towards boron compounds in order to derive the fine line between boron toxicity and tolerance among relevant bacteria population and to estimate any possible diversities within the tolerant species. The fifth element, boron (B), is crucial for maintaining healthy plant growth without seriously harming the biotic community of the surrounding soil. When boron is used excessively (attaining concentrations which can cause boron toxicity), tolerant bacteria that grew in the presence of boron up to a certain level may change or enhance their B-tolerance. To determine the precise boundary between boron toxicity and tolerance among the relevant bacterial population and to gauge any potential diversities within the tolerant species, boron-tolerant bacterial strains were isolated from soil samples with prolonged and chronic overexposure to boron compounds. Identification of diversity in boron tolerant bacteria would be done for the first time in North Bengal area with an avenue in further studying the possible mechanisms of tolerance development and probable methods for transfer of the genotypic factors or environmental causes alleviating tolerance levels. From the 16S rRNA based phylogenetic and biochemical analysis it was found that all the boron-tolerant bacterial strains isolated boron contaminated agricultural fields belongs to three different genera, *Lysinibacillus*, *Enterococcus* and *Bacillus*. A couple of boron tolerant strains, *Lysinibacillus* sp. strain OL1 and *Enterococcus* sp. strain OL5, found to be tolerate 230 mM and 210 mM boron maximally in form of boric acid respectively, were ultimately selected for in-depth study. They, OL1 and OL5, were also tolerant to several other heavy metals and metalloids, such as, arsenic, cadmium, copper, mercury, zinc and nickel. In comparison to other metal pollutants, boric acid [$B(OH)_3$] (approximately 96%) and a minor quantity of borate anion [$B(OH)^-_4$] are the two forms of boron that are the most mobile in soil. Both boric acid and borate can interact reversibly with different types of biomolecules like adenosine monophosphate, riboflavin, pyrimidine nucleotides, pyridoxine, ascorbic acid, and various sugar molecules (apiose, ribose, etc.) that contain an adjacent cis-hydroxyl group in their structure. Following growth kinetics and by measuring the amount of intracellular boron, it was primarily established that they were able to tolerate boron by using an inducible active efflux mechanism. This efflux mechanism enabled to maintain relatively less amount of boron in the intracellular milieu compared to the external B concentration. In order to validate the hypothesis, a potent efflux inhibitor PAβN was used; it was found that in the presence of PAβN (100 µg/ml) the level of B-tolerance got significantly reduced to 150 mM and 160 mM boron respectively, indicating that PAβN-sensitive-efflux pumps were partially responsible for B-tolerance. Three different omics tools, genomics, transcriptomis and proteomics, were used to generate data. Omics data were used to explore molecular mechanisms of boron tolerance in *Lysinibacillus* sp. OL1. RNA-free genomic DNA of OL1 was used for the construction of pair- end library for sequencing on NextSeq 500 system using Illumina platform. On the other hand, genomic DNA library of the strain OL5 was prepared using the Ion Xpress Plus fragment library kit (Thermo Fisher Scientific, USA) for Ion S5 next-generation sequencing platform (Thermo Fisher Scientific, USA). After annotation using NCBI Prokaryotic Genome Annotation Pipeline, it was found that the genome of OL1/OL5 isolates consisted of several number of efflux associated genes, 54/17 genes associated with MFS transporter, 263/283 genes associated with ABC transporter, 4/1 genes associated with SMR transporter, 2/1 genes associated with RND transporter and 4/2 genes associated with MATE transporter. Both the genomes were also containing a large number of genes associated with heavy metals and metalloids resistance. Total 33 and 30 heavy metals and metalloids resistance associated genes were present in the genome of OL1 and OL5 respectively. Beside all these, both OL1/OL5 genomes were found to contain at least 1/12 genes associated with phosphonate metabolism, 9/8 genes associated with auxin biosynthesis, 3/2 genes associated with siderophore or iron-siderophore transporter substrate binding, 8/2 genes associated with antioxidant activities, 78/40 genes associated with motility and chemotaxis, 6/39 genes associated with exo-polysaccharide production, and 7/ 23 genes associated with antimicrobial compounds and lytic enzymes production, which makes these two isolates a potential candidate for plant growth promoting rhizobacterial group. From the genomic and physiological analysis it was found that that OL1 have more genetic diversity and it can tolerate boron in more superior way compared to OL5. So, on the basis of these findings OL1 was selected for further studies. Total RNA and proteins were extracted from OL1 log phase growing culture according to standard methodology for transcriptomics and proteomics analysis. Following isolation, the proteome and transcriptome libraries were built and sequenced using the LC/MS and Illumina platforms, respectively. After the data had been sequenced and analysed, it was discovered that several stress response genes, efflux associated genes, energy metabolism, protein synthesis, and protein synthesis all played important roles in maintaining the integrity of the cell in the presence of an increased amount of extracellular boron. This result showed that multiple cellular metabolisms were involved in reducing the negative effects of boron on bacterial cells rather than just one. Furthermore, the most effective boron-tolerant isolate, OL1, has been subjected to *in-vivo* evolutionary engineering technique. It was discovered that OL-EC, an *in-vivo* evolutionary engineered cell, was able to tolerate 52.17% more boron than the OL1 wild type strain. The modified OL1-EC was able to withstand larger amounts of boron than the wild type of OL1, thus we tried to examine what mutations occurred in the OL1 genome and performed SNP-based mutational analysis. As a result of this analysis, we discovered that mutations occurred in a number of genes, including those encoding the ABC transporter ATP-binding protein, CPBP family intramembrane metalloprotease, transcription termination factor Rho, HEAT repeat domain-containing protein, GntR family transcriptional regulator, MarR family transcriptional regulator, oxidoreductase, FadR family transcriptional regulator, and a number of hypothetical proteins. We can infer from the results above that these advantageous mutations helped OL1-EC tolerate more boron. Future thorough studies utilising various omics methods, such as transcriptomics and proteomics, might give more details regarding the boron tolerance phenomenon in bacteria.

OL1 and OL5 were both found to be positive for IAA production, phosphate solubilization, lytic enzyme production, motility, and biofilm production; this indicates that these boron tolerant isolates can promote plant growth in boron rich bare land and restore the soil quality. The plant growth promotion properties of these two isolates were also characterized. Finally, a metagenomic technique was used to study the microbial diversity of soil supplemented with boron. For metagenomic analysis, the total metagenomic DNAs from soil samples that had been modified with boron and those that hadn't were collected. Metagenomic libraries were then created. Ion torrent was then used to sequence the libraries. OTUs were created from the sequenced data, and comparative analysis was done. It was revealed from the metagenomic data that the gram positive phylum, *Firmicutes* is prevalent along with other bacterial genera. Interestingly, all of the recovered boron-tolerant strains belonged to *Firmicutes*. Therefore, it's probable to infer that high level of boron stress lead to a selection pressure that encourages the growth of *Firmicutes*-related Gram-positive bacteria. Additionally, it was noted that the phyla *Acidobacteria*, *Actinobacteria*, and *Proteobacteria* are abundant following *Firmicutes*. The entire study was designed to support the hypothesis that there are a variety of microbes in the soil of North Bengal that can tolerate boron levels above normal and that these microbes have an impact on the biological interactions between boron, bacteria, and soil systems, which have an impact on agriculture, the environment, and people's health.