

Dedicated to...
Late Prof. Ashim Kumar Chakravarty
&
My Family

DECLARATION

I hereby declare that the research work incorporate in this thesis entitled “Revealing molecular genetics of boron tolerance/resistance in bacteria using *in-vivo* evolutionary engineering and high throughput tools” has been carried out by me in the Department of Biotechnology, University of North Bengal, Darjeeling-734013, West Bengal, India, under the supervision of Prof. (Dr.) Ranadhir Chakraborty, Department of Biotechnology, University of North Bengal, Darjeeling-734013, West Bengal, India. I also confirm that this work is original and has not been submitted before in part or full for any degree/diploma or any other academic award to this or any other University or Institution.

Place: NBU

Date: 12/12/2022

Subhajit Sen

Subhajit Sen, M.Sc.,
Senior Research Fellow,
Department of Biotechnology
University of North Bengal



UNIVERSITY OF NORTH BENGAL

DEPARTMENT OF BIOTECHNOLOGY
DARJEELING-734013, WEST BENGAL, INDIA

Prof. (Dr.) Ranadhir Chakraborty

CERTIFICATE

The research work presented in this thesis entitled “**Revealing molecular genetics of boron tolerance/resistance in bacteria using *in-vivo* evolutionary engineering and high throughput tools**” has been carried out under my direct supervision by **Mr. Subhajit Sen**. This work is original and has not been submitted for any degree or diploma to this or any other University or Institution.

Date: 12-12-2022


12-12-2022
(Prof. Ranadhir Chakraborty)

Supervisor
Ranadhir Chakraborty, Ph.D
Professor, Biotechnology
University of North Bengal
Siliguri- 734013, West Bengal

PLAGIARISM CHECK REPORT

Original
by Turnitin

Document Information

Analyzed document	Subhajit Sen_Biotechnology.pdf (D146961711)
Submitted	10/20/2022 9:01:00 AM
Submitted by	University of North Bengal
Submitter email	nbuplg@nbu.ac.in
Similarity	0%
Analysis address	nbuplg.nbu@analysis.arkund.com

Sources included in the report

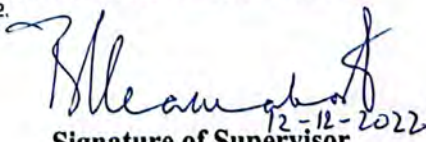
Entire Document

1 Revealing molecular genetics of boron tolerance/resistance in bacteria using in-vivo evolutionary engineering and high throughput tools A Thesis submitted to the University of North Bengal For the Award of Doctor of Philosophy in Biotechnology By Subhajit Sen Under the supervision of Prof. (Dr.) Ranadhir Chakraborty DEPARTMENT OF BIOTECHNOLOGY UNIVERSITY OF NORTH BENGAL November, 2022

2 Review of literature Boron, boron-based compounds, and isotopes Boron (B) is a group 13 (IIIA) metalloid element with atomic number (Z) = 5 and atomic mass (A) = 10.811 u. Borax (Na₂ [B₄ O₅ (OH)₄]·8H₂ O) and kernite (Na₂ [B₄ O₆ (OH)₂]·3H₂ O) are the major sources of natural boron. Environmental boron toxicity occurs in many parts of the world as a result of boron deposition in several natural exploits (Nable et al.1997). A relatively higher amount of boron is found in sedimentary rocks, soils, coal, and seawater. It was determined that the global average concentration of boron in seawater was approximately 4.6 mg/lit (Samman et al. 1998). A substantial proportion (7 – 18%) of boron in the environment comes from fertilizers, wastewater treatment plant releases, and fly ash waste released by coal-fired power plants (Raja and Omine 2013). About sixty-five to eighty-five percent of boron in the environment is derived from the world's oceans (Argust 1998). Boron is a constituent of several manufactured goods, such as glass, detergents, ceramics, and fertilizers, and may get in touch with the environment at the time of their production as a waste product released by the production plants (Argust 1998). Fruits (avocado, raisins, grape, apples, oranges, banana, etc.), vegetables (cauliflower, broccoli, carrots, spinach, lettuce, corn, etc.), and hazelnuts are known to be primary sources of boron (Hunt et al. 1991). Among vegetables, leafy greens contain the highest levels of boron compared to others, especially when they are grown without any chemical fertilizers (Newnham 1977). Vegetables, fruits, legumes, and tubers have much higher amounts of boron compared to grasses (e.g., wheat, rice, and corn retain 0.2 mg/kg) (Nielsen 1988; Vanderpool and Johnson 1992). Dried legumes, fruits, avocados, and nuts contain a minimum of 1.0 mg to a maximum of 4.5 mg boron/100 g (Naghii 1999). Fresh fruits, vegetables, honey, and bee pollen contain a minimum of 0.1 to a maximum of 0.6 mg boron/100 g, whereas foods from animal sources contain a relatively lower amount of boron at levels between 0.01 and 0.06 mg/100 g (Newnham 1977; Naghii 1999). In nature, two stable isotopes of boron are found, one is ¹⁰B and another one is ¹¹B. Both the isotopes have stable boron nuclei and are active in nuclear magnetic resonance (NMR) spectroscopy.

3 Few boron-containing natural products include the boric acid-based ionophoric macrodiolide antibiotics boromycin, borophycin, aplasmomycins A, B, and C, and tartrolons B, C, and E as shown in Fig. 1. Figure 1: Structures of boron-containing natural products Organoboron compounds are another type of boron-based compound that contains at least one carbon-to-boron bond. According to their structure, organoboron compounds are classified into several groups such as boranes, borinic acids, borinic esters, boronic acids, boronic (boronate) esters, boronamides, boryl anions, borate anions, and borohydrides. Also, other types of boron compounds are found, which are not technically organoboron compounds, they are utilized in synthesis as well. These compounds include borate (boric) esters and boron trihalides (containing three halogens on boron) as shown in Fig. 2.

Subhajit Sen
12/12/2022
Signature of Research Scholar
(Subhajit Sen)


Signature of Supervisor
(Prof. Ranadhir Chakraborty)
Ranadhir Chakraborty, Ph.D.
Professor, Biotechnology
University of North Bengal
Siliguri- 734013, West Bengal

PREFACE

In order to find answers to some fundamental questions in environmental microbiology, the OMICS laboratory of the Department of Biotechnology at the University of North Bengal was established. These questions included the effects of antibiotic use in human and veterinary medicine on microbial communities in freshwater and soil environments, the roles of host-associated and environmental factors in determining the composition of microbial communities in earthworms and facultative air-breathing fish, bacterial mechanisms to tolerate heavy metals, and microbial diversity in extreme environments.

Due to the fact that heavy metals are more frequently found in microbial habitats as a result of natural and man-made processes, microbes have developed a number of strategies to deal with their presence (either through efflux, complexation, or reduction of metal ions, or by using them as terminal electron acceptors in anaerobic respiration). Tolerance mechanisms have so far been identified and thoroughly described for metals like copper, zinc, arsenic, chromium, cadmium, and nickel. The OMICS laboratory has historically contributed to the study of the resistance of river-water bacteria to nickel, copper, cobalt, and zinc.

We have known about boron, a black or brown semiconducting metalloid, since antiquity (the name comes from the mineral borax). In 1808, the Frenchmen Gay-Lussac and Thenard as well as the Englishman Davy extracted boron from borax. It is the element with the highest tensile strength. Borates and other substances were utilized in the mummification process in ancient Egypt. Use of borax perborate as bleaching agents is known for long. Because ants and cockroaches cannot smell boric acid, it works well as an insecticide when combined with bait. Interestingly, boron is necessary for the development of cell walls in plants. In actuality, pollen tubes cannot be made without boron. The leaves also develop cracks, stains, and distortions. It has been used in Greece and Spain to revitalise old olive trees when mixed with pectin. Although boron is necessary for many organisms to carry out various physiological processes, it also becomes toxic after a certain threshold level. However, some bacteria can tolerate boron at levels above the threshold, although the exact mechanism by which they are able to do so is still unknown. These findings led us to start looking into the genetic processes underlying bacterial boron tolerance and resistance.

The primary goal of this study was to reveal the nature of boron tolerance in the bacterial strains that were isolated from a boron-contaminated cauliflower plantation

agricultural field. Physiological experiments and omics data were used to investigate the underlying molecular mechanism of boron tolerance. Using both traditional and cutting-edge phylogenomic tools, the boron-tolerant isolates were taxonomically characterised and their distinct taxonomic positions were established. For a better understanding of the molecular mechanism of boron tolerance, an *in-vivo* evolutionary engineering approach was tried for *Lysinibacillus* sp. OL1, the isolate with the highest boron tolerance. It was discovered that OL1 (OL1-EC) cells created through evolutionary-engineering were able to tolerate relatively more boron than the wild type bacteria. On the basis of these findings, it was hypothesised that certain genes may have provided selective advantage in tolerating relatively high amounts of boron without impairing its normal growth characteristics. This was confirmed by the detailed comparative genomic analysis of OL1 and OL1-EC. In order to determine the presence and function of boron-tolerant bacteria in soils contaminated with high levels of boron fertilisers, culture independent metagenome based studies were also carried out.

ACKNOWLEDGEMENT

It is with great respect I express my deep sense of gratitude and indebtedness to my supervisor **Prof. Ranadhir Chakraborty**, Professor, Department of Biotechnology, University of North Bengal for his expert advice, valuable suggestions, inspiring guidance, enthusiastic approach, constructive criticisms, thought provoking discussions, unreserved help and kind concern during the conduct of this research work and preparation of thesis. I value his knowledge and wisdom which nurtured this research in right direction without which fulfillment of this endeavor would not have been possible. I have always wanted to work with him, and completing my thesis successfully under his motivating direction has been a dream come true moment for me. He mentored me in both academic and extracurricular pursuits, and he is always a source of encouragement. He has supported me during each step of this venture and my obligation to him lasts forever. I really consider it my greatest fortune in having his guidance for my research work. I also want to express my gratitude to our Madam **Dr. Ratna Sarkar** for always being gracious and kind to us.

I would like to express my deepest gratitude to Late **Prof. Ashim Kumar Chakravarty** for his constant encouragement, kind behavior and blessings during my Ph.D. journey. I have been greatly inspired by his cheerful and upbeat attitude in the face of challenging situations. The emptiness after his sad demise cannot be described in words. But, I know that he will continue to bestow his blessings from wherever he is.

It is with immense pleasure, I express my whole hearted gratitude and never ending indebtedness to all the faculty members of Department of Biotechnology. Firstly, I would like to express my heartfelt thanks to **Dr. Anoop Kumar**, Head, Department of Biotechnology for always providing the unconditional help and encouraging guidance. I am highly grateful to him for giving me the opportunity to work with human cell lines in my early research phase.

I am also grateful to **Prof. Shilpi Ghosh** madam for guiding me so well in the beginning of my research carrier. Her expertise and unwavering encouragement have made me a motivated learner. She has always extended her kind help to me whenever I needed that.

Acknowledgement

I would also like to thank **Prof. Dipanwita Saha** and **Dr. Manab Deb Adhikari** for their enthusiastic support, constructive suggestions and immense blessings during my research work.

I thankfully acknowledge **Dr. Wriddhiman Ghosh**, Associate Professor, Department of Microbiology, Bose Institute, Kolkata, **Dr. Chayan Roy** and my friend **Mr. Nibendu Mandal** for their esteemed advice, timely help, and valuable suggestions throughout my research work.

I wish to express my special thanks to all the research scholars of **OMICS** laboratory, **Dr. Shriparna Mukherjee**, **Dr. Bipranish Kumar Tiwary**, **Dr. Tilak Saha**, **Dr. Rudra Prasad Roy**, **Mr. Subhanil Chakraborty**, **Dr. Vivek Kumar Ranjan**, **Mr. Sudipta Das**, **Ms. Chandana Basak**, **Mr. Partha Barman**, **Mrs. Sriradha Ganguly**, **Mr. Soumya Chatterjee**, **Ms. Anasuya Karjee**, **Mr. Anukul Paul**, **Ms. Shilpa Sinha**, **Mr. Deep Deb**. I feel very lucky to have seniors like **Subhanil da** and **Sudipta da** who were always with me during every ups and downs and helped me selflessly. Also, the group of juniors also deserves my special thanks as they not only assisted me in routine experiments but also in overcoming stressful situations. I am also thankful to our lab attendant cum my beloved friend **Mr. Arup Ghosh** and **Mr. Dipak Das** for their help and co-operation during my entire research work. Also, I want to thank M.Sc. dissertation student **Nidhi** for her valuable inputs. They had all contributed to make this trip more exciting and unforgettable than I could have ever anticipated. They are like my extended family, and I will treasure every second I spend with them.

My heartfelt thanks to all the other **research scholars** and non-teaching members of Department of Biotechnology, University of North Bengal. I am thankful to Late **Mr. Sujoy Chowdhury**, and **Mr. Raja Bauri** for their help and co-operation during my research work.

I would also like to convey my sincere thanks to our honorable present vice chancellor **Prof. Om Prakash Mishra**, Registrar **Prof. Pranab Ghosh**, and Dean of Science **Prof. Subhash Chandra Roy** for granting me the privilege to carry out my research work in this University.

I owe special thanks to **Department of Science and Technology (DST)**, Government of India for giving me the received research grants through **INSPIRE Fellowship** (vide sanction order **DST/INSPIRE Fellowship/2016/IF160786**) for smooth conduction of my research work.

Words fail when I express my feelings to my loving "Maa" Mrs. Sikha Sen and "Baba" Mr. Mrinal Sen without their support, prayers, blessings and sacrifices I would not have completed this work. No words can express my sincere gratitude towards my other family members and all my relatives for their love, incessant inspiration and constant prayers which helped me to complete this venture successfully.

Above all I humbly bow my head before the "LORD ALMIGHTY" whose grace had endowed me the inner strength and confidence, blessed me with a helping hand to complete this work successfully.

Lastly, I apologies for not having the opportunity to personally thank everyone who contributed to the effective completion of this thesis.

Date : 12/12/2022

Place: NBU

Subhajit Sen
(Subhajit Sen)

LIST OF TABLES

Table No.	Title of Table	Page No.
Table 1 :	Reported boron tolerant bacteria & their tolerance level	6 - 7
Table 1.1 :	Details of the soil sampling sites along with physicochemical properties.	26 - 27
Table 1.2 :	Different bacterial strains isolated from distinct boron-fertilizer-amended cauliflower cultivation fields of Maynaguri, West Bengal, India	27
Table 1.3 :	Accession no. of 16S rRNA gene sequence of different bacterial strains isolated from distinct boron-fertilizer-amended cauliflower cultivation fields of Maynaguri, West Bengal, India.	31
Table 1.4 :	Comparative colony morphological characteristics of <i>Lysinibacillus</i> sp. strain OL1 and <i>Enterococcus</i> sp. strain OL5 with closely related bacterial strains.	36 - 37
Table 1.5 :	Comparative biochemical characteristics of <i>Lysinibacillus</i> sp. strain OL1 and <i>Enterococcus</i> sp. strain OL5 with closely related bacterial strains.	39 - 43
Table 1.6 :	Heavy metals and metalloids tolerance level of the isolated boron tolerant bacteria <i>Lysinibacillus</i> sp. OL1 and <i>Enterococcus</i> sp. OL5.	45
Table 2.1 :	Intra-cellular boron concentration of OL1 and OL5 pre-exposed to different concentrations of boric acid and then incubated for 15 min in the presence of 200 mM boric acid.	65
Table 3.1 :	Quantitative analysis of genomic DNA isolated from OL1 and OL5	81
Table 3.2 :	Genomic features and accession numbers of the isolates <i>Lysinibacillus</i> sp. strain OL1 and <i>Enterococcus</i> sp. strain OL5	82 - 83
Table 3.3 :	Genome comparisons of boron tolerant isolates <i>Lysinibacillus</i> sp. OL1 with different closely related <i>Lysinibacillus</i> type strains (A) and <i>Enterococcus</i> sp. OL5 with different closely related <i>Enterococcus</i> type strains (B) in terms of G+C content, In silico DNA:DNA hybridization (DDH) (%) and Average Nucleotide Identity (ANI) (%)	86 - 87
Table 3.4 :	Quantification of isolated RNA samples using NanoDrop	92

List of tables

Table No.	Title of Table	Page No.
Table 3.5 :	High quality read statistics	94
Table 3.6 :	MCODE analysis of PPI network	109
Table 4.1 :	Boron tolerance level of purified evolutionary engineered <i>Lysinibacillus</i> sp. OL1	123
Table 4.2 :	Intra-cellular boron concentration of OL1 and OL1-EC exposed to 200 mM and 350 mM of boric acid for 15 min.	125
Table 4.3 :	Quantitative analysis of genomic DNA isolated from OL1-EC	126
Table 4.4 :	Genomic features and accession numbers of <i>Lysinibacillus</i> sp. strain OL1_EC	126 - 127
Table 4.5 :	Genome comparisons of <i>wild type</i> <i>Lysinibacillus</i> sp. OL1 with evolutionary engineered <i>Lysinibacillus</i> sp. OL1-EC, <i>In silico</i> DNA : DNA hybridization (DDH) (%) and Average Nucleotide Identity (ANI) (%)	128
Table 4.6 :	Single nucleotide substitutions detected across the completely aligned segments of the OL1 and OL1-EC genomes.	131 - 132
Table 4.7 :	Frameshift mutations detected across the completely aligned segments of the OL1 and OL1-EC genomes	133 - 134
Table 5.1 :	Average temperature, humidity, and rainfall of the experiment site.	137 - 138
Table 5.2 :	Summary statistics of the OTU analysis of soil samples	141
Table 5.3 :	Details of the soil sampling sites along with physicochemical properties.	144

List of tables in Appendix

Table A3.1 :	Efflux associated proteins present in genome of OL1 and OL5 based on automated proteins coding sequences through PGAP of NCBI.	208 - 218
Table A3.2 :	Genes associated with heavy metals and metalloids tolerance present in the genome of boron tolerant isolates OL1 and OL5.	218 - 220
Table A3.3 :	Genes associated with metabolic activities that are potentially related to plant growth promotion present in the genome of boron tolerant isolates OL1 and OL5.	220 - 233

Table No.	Title of Table	Page No.
Table A3.4 :	List of the upregulated DEGs (A) and proteins (B) involved in glycolysis / gluconeogenesis in <i>Lysinibacillus</i> sp. strain OL1 response to boron	234
Table A3.5 :	List of the upregulated DEGs (A) and proteins (B) involved in citrate cycle (TCA cycle) in <i>Lysinibacillus</i> sp. strain OL1 response to boron	235 - 236
Table A3.6 :	List of the upregulated DEGs (A) and proteins (B) involved in oxidative phosphorylation pathway in <i>Lysinibacillus</i> sp. strain OL1 response to boron	237 - 238
Table A3.7 :	List of the upregulated DEGs involved in pentose phosphate pathway in <i>Lysinibacillus</i> sp. strain OL1 response to boron	239
Table A3.8 :	List of the upregulated DEGs (A) and protein (B) involved in DNA replication in <i>Lysinibacillus</i> sp. strain OL1 response to boron	239 - 241
Table A3.9 :	List of the upregulated DEGs (A) and proteins (B) involved in transcription in <i>Lysinibacillus</i> sp. strain OL1 response to boron	241 - 244
Table A3.10 :	List of the upregulated DEGs (A) and protein (B) involved in translation in <i>Lysinibacillus</i> sp. strain OL1 response to boron	245 - 253
Table A3.11 :	List of the upregulated DEGs (A) and proteins (B) involved in efflux in <i>Lysinibacillus</i> sp. strain OL1 response to boron	253 - 257
Table A3.12 :	List of the upregulated DEGs (A) and protein (B) involved in stress response in <i>Lysinibacillus</i> sp. strain OL1 response to boron	257 - 260
Table A3.13 :	List of the upregulated DEGs (A) and proteins (B) involved in osmoregulation in <i>Lysinibacillus</i> sp. strain OL1 response to boron	260 - 261
Table A3.14 :	List of the upregulated DEGs (A) and protein (B) involved in pyruvate metabolism in <i>Lysinibacillus</i> sp. strain OL1 response to boron	261 - 263
Table A3.15 :	List of the upregulated DEGs (A) and protein (B) involved in propanoate metabolism in <i>Lysinibacillus</i> sp. strain OL1 response to boron	263 - 265
Table A3.16 :	List of the upregulated DEGs (A) and protein (B) involved in carbon fixation in <i>Lysinibacillus</i> sp. strain OL1 response to boron	265 - 266

Table No.	Title of Table	Page No.
Table A3.17 :	List of the upregulated DEGs (A) and protein (B) involved in purine metabolism in <i>Lysinibacillus</i> sp. strain OL1 response to boron	266 - 268
Table A3.18 :	List of the upregulated DEGs involved in pyrimidine metabolism in <i>Lysinibacillus</i> sp. strain OL1 response to boron	268 - 269
Table A3.19 :	List of the upregulated DEGs (A) and protein (B) involved in amino acid metabolism in <i>Lysinibacillus</i> sp. strain OL1 response to boron	269 - 272
Table A3.20 :	List of the upregulated DEGs (A) and proteins (B) involved in metabolism of cofactors and vitamins in <i>Lysinibacillus</i> sp. strain OL1 response to boron	272 - 276
Table A3.21 :	List of the upregulated DEGs (A) and proteins (B) involved in metabolism of terpenoids and polyketides in <i>Lysinibacillus</i> sp. strain OL1 response to boron	276 - 277
Table A3.22 :	List of other proteins upregulated and downregulated in <i>Lysinibacillus</i> sp. strain OL1 response to boron	277 - 280
Table A3.23 :	List of the significantly down-regulated DEGs in <i>Lysinibacillus</i> sp. strain OL1 response to boron	280 - 283
Table A3.24 :	Cluster associated pathways	284 - 285
Table A5.1 :	Phylum-level assortment of the total number of bacterial OTUs identified in MA1 and MA2 soil samples.	287
Table A5.2 :	Complete list of the genera identified in the MA1 and MA2 soil sample, along with the information about their presence or absence in the normal and boron amended soil samples, their Gram nature and their capabilities, if any, to tolerate boron.	288 - 302

LIST OF FIGURES

Figure No.	Figure Legends	Page No.
Figure 1 :	Structures of boron-containing natural products	2
Figure 2 :	Different types of organoboron compounds	2
Figure 1.1 :	Sampling site of soil: (A) Map view; (B) Satellite view	12
Figure 1.2 :	Schematic diagram of enrichment and isolation of boron tolerant bacteria	13
Figure 1.3 :	Polymerase chain reaction setup for full length 16S rRNA gene amplification	15
Figure 1.4 :	Polymerase chain reaction setup for full length 16S rRNA gene amplification from cloned cells.	16
Figure 1.5 :	Stock solution of heavy metals.	23
Figure 1.6 :	Geographical location of the sampling sites: (A) Map of India, showing the overview of sampling state (B) Map of West Bengal, showing the overview of the sampling sites. (C) Satellite view of the sampling sites MA2, MA3, FA4, SA5 and KD6 with exact geographical coordinate location.	26
Figure 1.7 :	Boron tolerance level of the bacterial isolates using different concentration of boric acid in the culture medium. Name of the isolates are plotted along the X-axis, while the boron tolerance level in terms of mM are plotted along the Y-axis. EC : <i>Escherichia coli</i> K12; BS : <i>Bacillus subtilis</i> .	28
Figure 1.8 :	Agarose gel electrophoresis of PCR-amplified 16S rRNA genes in different boron tolerant bacterial strains isolated from boron contaminated agricultural soil sample. Lane show positive products (nearly 1500 bp) of the 16S rRNA gene for isolates OL1, OL2, OL3, OL4, OL5, OL6, OL7, OL8, OL9, OL10, OL11 and OL12, M-Marker 500bp (A). Restriction fragment length polymorphism (RFLP) of the 16S rRNA gene of the isolates. Marker lane contains a 50 bp DNA ladder. There are two type of polymorphism observed; total 10 isolates (OL1, OL2, OL3, OL4, OL7, OL8, OL9, OL10, OL11, OL12) belongs to Group I and two isolates (OL5, OL6) belongs to Group II (B).	29 - 30
Figure 1.9 :	Phylogenetic tree showing inter relationships of the <i>Lysinibacillus</i> sp. strain OL1, OL2, OL3, OL4 and OL7 with the most closely related <i>Lysinibacillus</i> species inferred from the sequences of 16S rRNA gene.	33
Figure 1.10 :	Phylogenetic tree showing inter-relationship of the <i>Enterococcus</i> sp. strain OL5 and OL6 with the most closely related <i>Enterococcus</i> species inferred from the sequences of 16S rRNA gene.	34

Figure No.	Figure Legends	Page No.
Figure 1.11 :	Phylogenetic tree showing inter relationships of the <i>Bacillus</i> sp. strain OL8, OL9, OL10, OL11 and OL12 with the most closely related <i>Bacillus</i> species inferred from the sequences of 16S rRNA gene.	35
Figure 1.12 :	Light and scanning electron micrographs of OL1 and OL5. micrographs were recorded at 1000X magnification while in the electron micrographs the scale bar represents 2 μ m length.	38
Figure 1.13 :	Growth optimization of the isolates OL1 and OL5 at different physiological parameters; (A) Temperature, (B) pH, (C) Nutrient concentration, (D) Salt concentration. All the experiments were done in triplicate and the standard deviations (SD) were plotted in the graphs as error bars.	44
Figure 1.14 :	PGPR attributes of the isolates OL1 and OL5; IAA production (A) OL1 and (B) OL5; and (C) phosphate solubilization activity. All the experiments were done in triplicate and the standard deviations (SD) were plotted in the graphs as error bars.	47
Figure 1.15 :	Biofilm formation assay of the isolates OL1 and OL5; (A) Crystal Violet stained microplate plates and (B) Quantification of crystal violet dye attached to the cells forming biofilms on microplate plates. All the experiments were done in triplicate and the standard deviations (SD) were plotted in the graphs as error bars.	48
Figure 2.1 :	Growth of (A and C) OL1 and (B and D) OL5 in the absence (A and B) and presence (C and D) of 200 mM boric acid. All the values plotted are the averages of three different experiments; the standard deviations of the data have been shown in the form of error bars.	59
Figure 2.2 :	Growth behavior of the isolates in TSB supplemented with 200 mM boric acid after pre-exposure to 0-50 mM boric acid: (A) OL1 and (B) OL5, as recorded at intervals of 4 h; (C) OL1 and (D) OL5, as recorded at intervals of 1 h. All the values plotted are the averages of three different experiments; the standard deviations of the data have been shown in the form of error bars.	60
Figure 2.3 :	Growth and survivability of boron tolerant isolates under different oligotrophic conditions: (A) OL1 and (B) OL5 in different strengths of R2A medium; (C) OL1 and (D) OL5 in different strengths of TSB medium. Different time intervals (day) are plotted along the X-axis, while the bacterial cell count (log CFU /ml) is plotted along the Y-axis. All the experiments were done in triplicate and the standard deviations (SD) were plotted in the graphs as error bars.	62

Figure No.	Figure Legends	Page No.
Figure 2.4 :	Quantification of extracellular, intracellular, and cell-bound boron after 24, 48, and 72 h of incubation in TSB supplemented with 100 mM boric acid: (A) OL1 and (B) OL5. The values plotted are averages of three different experiments; the standard deviations of the data have been shown in the form of error bars.	64
Figure 2.5 :	Growth of OL1 and OL5 in presence of different concentrations of PAβN in TSB medium. The values plotted are averages of three different experiments; the standard deviations of the data have been shown in the form of error bars.	66
Figure 2.6 :	Maximum tolerable concentrations of boric acid for OL1 and OL5 in TSB, or TSB plus PAβN. The values plotted are averages of three different experiments; the standard deviations of the data have been shown in the form of error bars.	67
Figure 3.1 :	Bioinformatics workflow of transcriptome analysis	77
Figure 3.2 :	Qualitative analysis of genomic DNA isolated from <i>Lysinibacillus</i> sp. strain OL1 and <i>Enterococcus</i> sp. strain OL5	81
Figure 3.3 :	Phylogenetic tree inferred by Species Tree Builder. (A) <i>Lysinibacillus</i> sp. strain OL1 with the most closely related <i>Lysinibacillus</i> species, (B) <i>Enterococcus</i> sp. strain OL5 with the most closely related <i>Enterococcus</i> species inferred from the sequences of a set of 49 core, universal genes defined by COG (Clusters of Orthologous Groups) gene families.	84 - 85
Figure 3.4 :	RAST annotation report of both the boron tolerant isolates, OL1 (A) and OL5 (B)	89 - 90
Figure 3.5 :	Isolated RNA from the Control (OL1_C) and Experimental (OL1_T) conditions and samples, run on on 1% denatured Agarose gel.	92
Figure 3.6 :	Library profile of sample OL1_C (A) and OL1_T (B) on Agilent Tape Station using high sensitivity D1000 screen tape.	93
Figure 3.7 :	KEGG pathway analysis; significantly enriched KEGG pathway in <i>Lysinibacillus</i> sp. strain OL1 in response to boron.	95
Figure 3.8 :	Significantly upregulated gene expression profile associated with carbohydrate metabolism in OL1 in presence of boron. Heat map was constructed using the FPKM value of both control and treated condition.	98
Figure 3.9 :	Significantly upregulated gene expression profile associated with energy metabolism in OL1 in presence of boron. Heat map was constructed using the FPKM value of both control and treated condition.	98

Figure No.	Figure Legends	Page No.
Figure 3.10 :	Significantly upregulated gene expression profile associated with stress response in OL1 in presence of boron. Heat map was constructed using the FPKM value of both control and treated condition.	99
Figure 3.11 :	Significantly upregulated gene expression profile associated with efflux transporters in OL1 in presence of boron. Heat map was constructed using the FPKM value of both control and treated condition.	100
Figure 3.12 :	Significantly upregulated gene expression profile associated with genetic information processing in OL1 in presence of boron. Heat map was constructed using the FPKM value of both control and treated condition.	101
Figure 3.13 :	Significantly upregulated gene expression profile associated with nucleotide metabolism in OL1 in presence of boron. Heat map was constructed using the FPKM value of both control and treated condition.	102
Figure 3.14 :	Significantly upregulated gene expression profile associated with amino acid metabolism in OL1 in presence of boron. Heat map was constructed using the FPKM value of both control and treated condition.	102
Figure 3.15 :	Significantly upregulated gene expression profile associated with metabolism of terpenoids and polyketides in OL1 in presence of boron. Heat map was constructed using the FPKM value of both control and treated condition.	103
Figure 3.16 :	Significantly upregulated gene expression profile associated with Cofactor and vitamins metabolism in OL1 in presence of boron. Heat map was constructed using the FPKM value of both control and treated condition.	103
Figure 3.17 :	Mass spectroscopic analysis of proteins isolated from boron amended (red) and unamended (black) conditions.	104
Figure 3.18 :	Heat map created on the basis of total number of protein per pathway detected from <i>Lysinibacillus</i> sp. strain OL1 using LC-MS in presence and absence of boron.	107
Figure 3.19 :	Protein-protein interaction network constructed using STRING (Search Tool for the Retrieval of Interacting Genes; version 11.5) with significantly upregulated DEGs in <i>Lysinibacillus</i> sp. strain OL1 response to boron.	108
Figure 3.20 :	Cluster generation with all the upregulated DEGs using MCODE.	109
Figure 3.21 :	Constructed pathway network from cluster 1.	110
Figure 3.22 :	Constructed pathway network from cluster 2 (A) and cluster 3 (B).	111
Figure 3.23 :	Constructed pathway network from cluster 4.	111

Figure No.	Figure Legends	Page No.
Figure 3.24:	Constructed pathway network from cluster 5 (A) and cluster 6 (B).	112
Figure 3.25 :	Venn diagram of the identified hub genes	113
Figure 3.26 :	Hypothesized model of boron tolerance by <i>Lysinibacillus</i> sp. strain OL1	114
Figure 4.1 :	General methods of evolutionary engineering	116
Figure 4.2 :	Flow chart of <i>In-vivo</i> evolutionary engineering approach we have applied	118
Figure 4.3 :	Growth behavior of the isolates OL1 and OL1 EC in TSB (A), TSB supplemented with 200 mM boric acid (B) and TSB supplemented with 350 mM boric acid (C). All the values plotted are the averages of three different experiments; the standard deviations of the data have been shown in the form of error bars.	124
Figure 4.4 :	Qualitative analysis of genomic DNA isolated from <i>Lysinibacillus</i> sp. strain OL1-EC	125
Figure 4.5 :	Phylogenetic tree inferred by Species Tree Builder. <i>Lysinibacillus</i> sp. strain OL1-EC and OL1 with the most closely related <i>Lysinibacillus</i> species, inferred from the sequences of set of 49 core, universal genes defined by COG (Clusters of Orthologous Groups) gene families	129
Figure 5.1 :	Graphical representation of the flow of experiments (with representative results). Soil panels: (A) unamended soil and (B) boron amended soil.	136
Figure 5.2 :	Polymerase chain reaction setup for V3 region of 16S rRNA gene amplification	139
Figure 5.3 :	Bubble plot comparing the temperature, humidity and rain fall of the different months when the experiment was carried out. Humidity is plotted along the X-axis, temperature along the Y-axis, and individual months are represented by different color circles. The size of the circles is proportional to the amount of rainfall.	145
Figure 5.4 :	The type of natural flora was observed in the MA1 (boron unamended soil) and MA2 (boron amended soil) samples on different days throughout the experiment.	147
Figure 5.5 :	Rarefaction curves showing the proportionality between OTU-level diversity revealed and the number of amplified 16S rRNA gene sequence (V3 region) reads analyzed for the individual soil samples collected from MA1 (boron unamended soil) and MA2 (boron amended soil). For each sample, a number of reads used in OTU-clustering are plotted along the X-axis, while species count is plotted along the Y-axis.	149

Figure No.	Figure Legends	Page No.
Figure 5.6 :	Microbial community in MA1 (boron unamended soil) and MA2 (boron amended soil) on different days of the experiment; phylum level distribution of the OTUs was plotted here.	151
Figure 5.7 :	Venn diagram displaying the common and unique genera among all six (boron amended and unamended) soil sample microbial communities.	154
Figure 5.8 :	Principal coordinate analysis plot of all samples between boron amended (represent as MA2) and unamended (represent as MA1) soil. Each dot represents one sample of a different day throughout the experiment.	155
Figure 5.9 :	Differential abundance analysis of some class (A), order (B), family (C, D), and genus (E,F,G,H,I) of bacteria found in different soil samples.	157 - 161

List of figures in Appendix

Figure A1.1 :	Agarose gel electrophoresis of isolated genomic DNA of all boron tolerant isolates.	205
Figure A1.2 :	Agarose gel electrophoresis of PCR-amplified 16S rRNA genes in different boron tolerant bacterial strains isolated from boron contaminated agricultural soil sample.	206
Figure A2.1 :	Intracellular and extracellular boron content determination, (A) solution before incubation; (B) solution after incubation.	207
Figure A4.1 :	Intracellular boron content determination, B – blank; OL1 – wild type OL1 cells; OL1-EC – <i>in- vivo</i> evolutionary engineered OL1 cells.	286
Figure A5.1 :	Relative abundance of bacteria (genus level) in boron amended (marked as BT) and unamended soil samples (marked as BUnT).	303 - 308

ABBREVIATIONS

Abbreviation	Description
ABC	ATP-binding cassette
ANI	Average nucleotide identity
BacMet	Biocide and Metal Resistance Genes Database
B	Boron
BSA	Bovine serum albumin
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cm	Centimeter
°C	Degree Celsius
dsDNA	Double stranded deoxyribonucleic acid
CFU	Colony-forming unit
cDNA	Complementary deoxyribonucleic acid
COG	Clusters of Orthologous Groups
CTAB	Cetrimonium bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DGE	Differential gene expression
DMSO	Dimethyl sulfoxide
DDH	DNA:DNA hybridization
EMS	Ethyl methane sulfonate
EDTA	Ethylenediamine tetraacetic acid
FPKM	Fragments per kilobase of exon per million mapped fragments
gDNA	Genomic DNA
GO	Gene Ontology
GGDC	Genome-to-Genome Distance Calculator
GPS	Global Positioning System

Abbreviations

Abbreviation	Description
g	Gram
h	Hour(s)
IAA	Indole acetic acid
Kb	Kilobase pair
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS	Liquid chromatography mass spectrometry
MFS	Major facilitator superfamily
MR/VP	Methyl Red / Voges-Proskauer
min	Minute (s)
mm	Millimetre
mg	Milligrams
ml	Milliliters
MG-RAST	Metagenomic Rapid Annotations using Subsystems Technology
MIC	Minimum inhibitory concentration
M	Molar
MEGA	Molecular Evolutionary Genetics Analysis
mM	Millimolar
μl	Microliter
μg	Microgram
Mmol/lit	Millimoles per litre
MSA	Multiple Sequence Alignment
MATE	Multi antimicrobial extrusion
ng	Nanogram
N	Normality
nm	Nanometre
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance

Abbreviation	Description
NGS	Next-generation sequencing
OD	Optical density
OTU	Operational Taxonomic Unit
ORF	Open reading frame
ppm	Parts per million
PGM	Personal Genome Machine
PA β N	Phenylalanine-arginine β -naphthylamide
PBS	Phosphate-buffered saline
PSI	Phosphate solubilization index
PCR	Polymerase chain reaction
PGAP	Prokaryotic Genome Annotation Pipeline
PPI	Protein-protein interaction
QV	Quality values
RAST	Rapid Annotation using Subsystem Technology
Rpm	Rotation per minute
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RFLP	Restriction fragment length polymorphisms
R2A	Reasoner's 2A
RND	Resistance nodulation-cell division
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
sec	Second (s)
SRA	Sequence Read Archive
SEM	Scanning electron microscope
SNPs	Single nucleotide polymorphisms

Abbreviations

Abbreviation	Description
SMR	Small multidrug resistance
SPAdes	St. Petersburg genome assembler
NaCl	Sodium chloride
TAE	Tris acetate EDTA
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TG	Triglyceride
UV	Ultraviolet
VLDL	Very low density lipoprotein
v/v	volume/volume
w/v	weight/volume
WHO	World health organization
WGS	Whole-genome sequencing
