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

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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
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PLAGIARISM CHECK REPORT

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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 (Na 2 [B 4 O 5 (OH) 4]·8H 2 O) and kernite (Na 2 [B 4 O 6 (OH) 2]·3H 2 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 10 B and another one is 11 B. 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.

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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.

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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.

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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

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Subhajit Sen

(Subhajit Sen)

LIST OF TABLES

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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
^{o}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

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
PAβN	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

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