Review of the literature illuminating the context supporting the research's goals and hypothesis

I. 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 ($Na_2[B_4O_5(OH)_4]\cdot 8H_2O$) and kernite ($Na_2[B_4O_6(OH)_2]\cdot 3H_2O$) 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 considerable 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.

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.

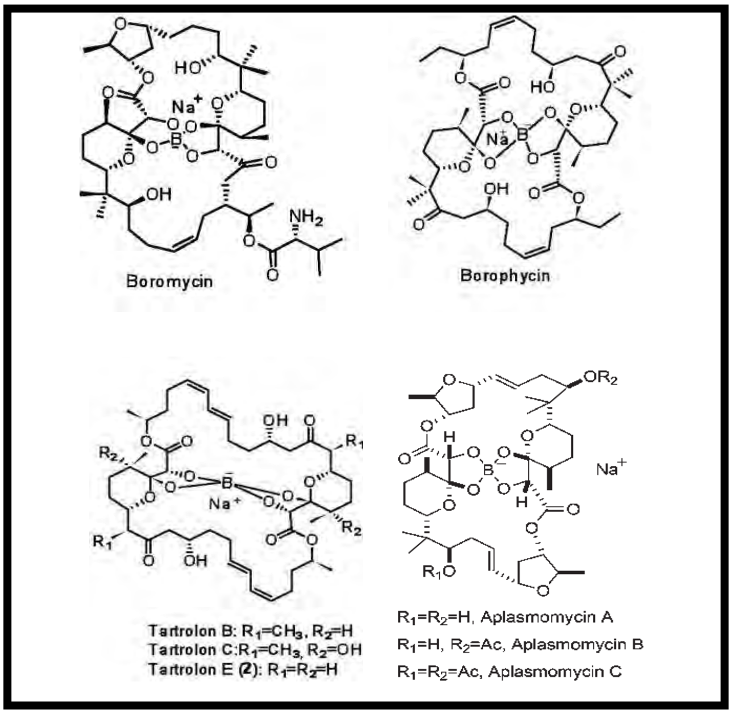

Figure 1 : Structures of boron-containing natural products

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.

BR_3 boranes	$BR_2(OH)$ borinic acids	$BR_2(OR)$ borinic esters	$BR(OH)_2$ boronic acids
$BR(OR)_2$ boronic (boronate) esters	$B(OH)_3$ boric acid	$B(OR)_3$ borate (boric) esters	$RB(NR_2)_2$ boronamides
BX_3 boron trihalides	$[:BR_2]^-$ boryl anions	$[R_3BH_3]^-$ borohydrides	$[RBF_3]^-$ trifluoroborates

Figure 2 : Different types of organoboron compounds

Boric acid is practically a non-toxic compound and also it is a metabolic end-product of many organoboron compounds. Boric acid has an affinity for cis –hydroxyl groups in both *in-vitro* and *in- vivo* models, such affinity towards cis –hydroxyl groups may affect the biological systems and it becomes toxic to the living world after a certain threshold level

(World Health Organization 1998; Bolaños *et al.* 2004). Due to its inhibitory effects on microorganisms, compounds of boron are regularly used as a food preservative, surface disinfectant, and in the treatment of recurrent vulvovaginal candidiasis caused by some species of *Candida* and *Saccharomyces* (Swate and Weed 1974; Otero *et al*. 2002). Boron-based compounds are also used in fertilizer production, colouring material production, corrosion inhibitors, and pharmaceutical compound buffers production (Heather DeFrancesco *et al*. 2016).

II. Effect of boron in the living world

Recent studies suggest that boron has a broad range of effects on biological systems. Boron plays an important role in various metabolic, nutritional, hormonal, and physiological processes (Blevins and Lukaszewski 1998). From different studies, we have found that boron is not that much essential to plants (except for some high boron demanding plants such as cauliflower, alfalfa, sugar beets, potatoes, sunflower, soybeans, and canola), but boron is an essential micronutrient for humans and animals (Basoglu *et al*. 2000; Hunt 2012; Nielsen 1997; Kabu and Civelek 2012). In microorganisms too, boron plays several essential functions. But unfortunately, the mechanisms of boron transport and tolerance are still not clearly understood in bacteria.

II.A. *Effect on animals*

Literature has made it evident that boron is an essential micronutrient for humans and animals (Kabu and Civelek 2012; Nielsen 1997; Hunt 1994). It has a low atomic weight and can easily bind to different organic compounds, this property of boron enable it to influence biological functions (Hunt 1998). The level of boron present in organisms is very minimum, at this level it is nontoxic but essential (in boron deficiency some developmental abnormalities were reported) for the growth of an organism. Organisms have an innate ability to maintain the boron level in the body up to a certain threshold level (Hunt 1998).

From different studies, it was found that boron intake affects animals. According to Basoglu *et al*. 2002 and Bobe *et al*. 2004 sodium borate ($Na_2B_4O_7$) inhibits the development of fatty liver in cows and a significant decrease in serum triglyceride (TG) and very low-density lipoprotein (VLDL) levels were observed in sodium borate treated cows. From other studies, it was also found that sodium borate may help to improve the metabolic functions during the periparturient period and in cattle reduce the formation of the fatty liver during the early lactation period (Kabu and Civelek 2012; Basoglu *et al*. 2002). According to Basoglu *et* *al*. 2000, 2010, 2011 borax ($Na_2B_4O_7 \cdot 10H_2O$) was able to reduce the blood lipid level in New Zealand Rabbits and dogs, thus reducing the chance of formation of fatty liver. Different studies suggested that boron affects the glucose–alanine cycle, the Krebs cycle, and methionine metabolism as a result of this the oxidative stress was reduced and the lipid profile was positively affected (Basoglu *et al*. 2011). In other studies, it was observed that boron affects vitamin D_3 metabolism and helps to maintain the blood glucose level in the chicks (Hunt *et al*. 1983).

II.B. *Effect on humans*

Humans fulfill their boron demand via the consumption of food. The maximum amount of boron is found in fruits like apples, avocado, grapes, oranges, bananas, etc.; vegetables like broccoli, cauliflower, potatoes, tomatoes, etc.; legumes, pulses, and nuts. A certain amount of boron is also obtained from coffee and milk. In a healthy human being total boron concentration in blood found to be are in the range of 15.3–79.5 ng/g wet wt (Clark *et al*. 1987). Boron has been found in the human body in form of boric acid [$B(OH)_3$] (98.40%) and borate anion [$B(OH)^-_4$] (1.60%) (Sutherland *et al*. 1998; Nielsen 1997). Boron concentration in different body organs differs, which suggests that metabolic roles played by boron differ from organ to organ (Newnham 1991). Boron has high nutritional importance for humans and its plays a major role in different metabolic pathways in humans, for example, boron can affect the composition or function of the brain, immune systems, and skeletal muscles (Nielsen 1997). Dietary intake of boron helps humans in several different functions, such as arresting osteoporosis in postmenopausal women, excretion level of essential elements (magnesium and calcium) through urine also decreased, and boron supplementation also shows a similar effect to estrogen supplementation in women who suffer from postmenopausal osteoporosis, it also helps to increase the calcium ion level and 1,25-dihydroxycholecalciferol level of blood serum, and decrease serum calcitonin level (Nielsen *et al*. 1987; Nielsen 1990).

II.C. *Effect on plants*

Boron acts as an essential micronutrient in higher plants (Blevins and Lukaszewski 1998). It plays important role in different physiological functions of plants, such as cell wall synthesis and lignifications, maintenance of cell wall structure, respiration, carbohydrate metabolism, RNA metabolism, phenol metabolism, indole acetic acid metabolism, membrane transport, and sugar transport (Blevins and Lukaszewski 1994; Camacho-Cristóbal *et al*. 2008).

Vegetables, fruits, and hazelnuts are known to be major sources of boron (Hunt *et al.* 1991). Among all the vegetables, leafy greens are containing the highest amount of boron, mainly when they are cultivated in an organic way (Newnham 1977). Boron insufficiency in plants causes carbohydrate deposition in the chloroplasts of plants, makes the pentose phosphate cycle hyperactive and may slow down the activity of the Krebs cycle (Lovatt and Dugger 1984; Goldbach 1997). The available concentration of boron in the soil varies due to soil type, pH, and temperature. Also, the profound use of fertilizers can increase the boron concentration in soil (Newnham 1977).

II.D. *Effect on microorganisms*

Boron in form of borate ion shows effects on several bacteria and fungi (Hunt 2003; Rolshausen and Gubler 2005; Baker *et al*. 2009; Tamay-Cach *et al*. 2012). According to Hunt 2003, boron is an essential trace element for some microorganisms belonging to Stramenopila, Eubacteria, Viridiplantae, Animalia, and Fungi. Due to its inhibitory effects on microorganisms, compounds of boron are regularly used as food preservatives, surface disinfectants and in the treatment of recurrent vulvovaginal candidiasis caused by some species of *Candida* and *Saccharomyces* (Swate and Weed 1974; Otero *et al.* 2002). Boron is essential for *Azotobacter* (Anderson *et al*. 1961) and *Actinomycetes* of the genus *Frankia* (Bolanos *et al*. 2002). These specific microorganisms probably require boron for their envelope stability. Under nitrogen-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), but not required for the growth. In another report, a boron-based auto-inducer was characterized that could mediate quorum sensing in bacteria (Xin Chen *et al*. 2002). Antibiotics like boromycin and tartrolon A, synthesized by bacteria, are also known to contain boron. The fact remains that only a few microorganism species can grow in high boron concentration in natural environments. In *cyanobacteria* and *Bacillus boroniphilus* sp. nov. boron as boric acid has been found as an essential micronutrient for their growth (Mateo *et al*. 1986; Ahmed *et al*. 2007a). Boron resistance has been reported in several different bacterial genera such as *Algoriphagus*, *Arthrobacter*, *Bacillus*, *Chimaereicella*, *Gracilibacillus*, *Lysinibacillus,* and *Rhodococcus*, and attempts have been made to understand the process mechanistically (Ahmed *et al*. 2007a–d; Ahmed and Fujiwara, 2010).

III. Diversity of boron-tolerant bacteria

Boron is toxic to living cells when present above a certain threshold. Hence, bacteria that grow in the high boron-containing environment will be of immense significance for studying the underlying mechanism(s) to survive under high boron stress and also in identifying genetic or molecular mechanisms of tolerance/resistance. Recent investigations have shown newly discovered bacteria that can grow in the presence of boron produced from boric acid at concentrations of 50–450 mmol/L. These bacteria were isolated from regions with high boron concentrations.There are reports of boron-tolerant bacteria like *Bacillus boroniphilus* sp. nov. (Ahmed *et al*. 2007a) that required boron to grow and could tolerate more than 450 mM boric acid, *Gracilibacillus boraciitolerans* sp. nov. (Ahmed *et al*. 2007b) could tolerate up to 450 mM boric acid, *Chimaereicella boritolerans* sp. nov. (Ahmed *et al*. 2007c) could tolerate up to 300 mM boric acid, *Lysinibacillus boronitolerans* (Ahmed *et al*. 2007d) tolerated up to 150 mM boric acid, *Lysinibacillus parviboronicapiens* (Miwa *et al*. 2009a) tolerated up to 50 mM boric acid, etc. while growing under laboratory set-up. Details of all the reported boron-tolerant bacteria are shown in Table 1.

Table 1 : Reported boron tolerant bacteria & their tolerance level

Sl. No.	Reported boron tolerant bacteria	Tolerance level of boron	Reference
1.	*Bacillus boroniphillus* DSM 17376^T	450 mM	Ahmed *et al*. 2007
2.	*Lysinibacillus parviboronicapiens* BAM-582^T	50 mM	Miwa *et al*. 2009
3.	*Lysinibacillus boronitolerans* 10a^T	150 mM	Ahmed *et al*. 2007d
4.	*Lysinibacillus fusiformis*	60 mM	Priest *et al*. 1988
5.	*Lysinibacillus sphaericus*	75 mM	Claus and Berkeley 1986
6.	*Bacillus silvestris* DSM 12223^T	60 mM	Rheims *et al*. 1999
7.	*Bacillus firmus*	300 mM	Verce *et al*. 2012
8.	*Lysinibacillus fusiformis* M1	260 mM	Raja and Omine 2012
9.	*Bacillus cereus* M2 and M3	60 mM	
10.	*Bacillus pumilus* M4	180 mM	
11.	*Rhodococcus baikonurensis*	100 mM	Ahmed and Fujiwara 2010
12.	*Arthrobacter* sp.	80 mM	
13.	*Algoriphagus* sp.	300 mM	
14.	*Gracilibacillus* sp.	450 mM	
15.	*Microbacterium* sp. Cr-D	200 mM	Raja and Omine 2013

Sl. No.	Reported boron tolerant bacteria	Tolerance level of boron	Reference
16.	*Ralstonia* sp. FAI	100 mM	
17.	*Bacillus* sp. KU1, KU2, KU3, KU4, KU6, KU7, KU9, KU12, KU13, KU14, KU23, As14, As30 and Cr-50	100 – 260 mM	
18.	*Lysinibacillus* sp. KU22	220 mM	
19.	*Pseudomonas stutzeri* B2	100 mM	Nural Yaman *et al.* 2019
20.	*Enterococcus mundtii* B10	50 mM	
21.	*Acinetobacter* sp. B11, B16	50 mM	
22.	*Acinetobacter schindler* B14	50 mM	
23.	*Enterococcus faecalis* DB3	50 mM	
24.	*Bacillus sp.* DB4, DB14, DB23	50 mM	
25.	*Halomonas aidingensis* DB5	50 mM	
26.	*Bacillus* sp. NCCP-132, NCCP-133, NCCP-136	100 mM	Javed *et al*. 2017
27.	*Ornithinibacillus* sp. NCCP-134	100 mM	
28.	*Oceanobacillus* sp. NCCP-135	100 mM	
29.	*Bacillus* spp. MN-54	180 mM	Samreen *et al*. 2019

It was found that the majority of the reported boron-tolerant bacterial isolates were gram-positive across the board, with the exception of *Algoriphagus*, *Ralstonia*, *Pseudomonas*, *Acinetobacter*, and *Halomonas*, which are gram-negative.

IV. The probable habitat of boron-tolerant bacteria

Across the globe boron-tolerant bacteria were isolated from several different boron-contaminated environments such as, the soil of the Hisarcik area, in the Kutahya province of Turkey (Ahmed *et al*. 2007), fly ash dumping site of Nagasaki prefecture, Japan (Raja and Omine 2013), boron mining site of California (Verce *et al*. 2012), mining site of Hokkaido in Japan (Raja and Omine 2012) boron contaminated area of kirka, Eskişehir, Turkey (Nural Yaman *et al*. 2019), etc. Boron-tolerant bacteria were also isolated from the phyllosphere near the geothermal area (Baldi *et al*. 1995). A large number of boron-tolerant bacteria were isolated from the soil of Turkey because Turkey is known as the largest natural boron reservoir in the world. Boron tolerant bacteria were also found in exudates from different sewage treatment plants because the waste materials from these plants contain a relatively higher amount of boron, which selectively promotes the growth of boron tolerant bacteria. In different agricultural fields (mainly crops that required a relatively higher amount of boron for growth) an abundance of boron tolerant bacteria was also observed (this study).

V. The probable mechanism(s) of boron tolerance

In a different study conducted in plants, it has been found that the boron tolerance property in barley plants was achieved by maintaining low boron concentration in both roots and shoots (Nable *et al*. 1990; Hayes and Reid 2004; Sutton *et al*. 2007). According to Dordas and Brown 2000, boron transport across the plasma membrane can be affected by its permeability (boron transport across the plasma membrane depends upon the lipid composition of the membrane and it can be affected by the alteration in lipid composition). Several different studies reported that efflux is the main mechanism of boron tolerance in plants and yeast (Miwa *et al*. 2007; Kaya *et al*. 2009). Different types of boron transporters were reported in yeast and plants, such as DUR3, ATR1, FPS1, and BOR1 in *Saccharomyces cerevisiae*; NIP5 and PIP1A in *Arabidopsis thaliana* (Nozawa *et al*. 2006; Takano *et al*. 2002; Kaya *et al*. 2009). 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, the mechanisms of boron transport and tolerance in bacteria were still not clearly understood (Miwa and Fujiwara 2010). According to Ahmed and Fujiwara 2010, a negative correlation was found between cellular boron content and the environmental boron content in boron tolerant bacteria, suggesting boron efflux may be the major mechanism of tolerance.

Under these circumstances, the objectives of this study were formulated to prove or disprove the hypothesis and determine the possible mechanisms of boron tolerance in boron-tolerant bacteria using both classical microbiological methods and omics tools. In order to develop/select strains that would withstand more boron than the initial strain, an *in-vivo* evolutionary engineering approach was used to better understand the molecular mechanisms of boron-stress.

VI. Hypothesis

The intensity of the immediate damage that boron causes when it enters the bacterial cell cannot be tolerated by a single molecular event (like efflux); rather, the strain must have its genetic circuits rewired to charge up higher than the normal expression of genes for all fundamental macromolecule biosynthesis in addition to stress-reduction techniques.

VII. Objectives of the study

The present work had been undertaken with the following objectives-

1. Isolation and characterization of boron tolerant/resistant bacteria from boron-rich soil.
2. Standardization of microbiological method(s) to ascertain boron tolerance limit in defining boron tolerant/resistant bacteria.
3. Application of *in-vivo* evolutionary engineering principle to study the nature of boron tolerance in bacteria in greater details.
4. Revelation of the molecular basis of boron tolerance in a suitable boron tolerant/resistant isolate using omics data generated from genome, transcriptome, and proteome.
5. Metagenomic analyses (by Next Generation Sequencing) of the boron-amended soil to understand the effect of boron on soil bacterial diversity.