EFFECTS OF pH SHOCK AND HEAT STRESS ON THE GROWTH OF NITROGEN-FIXING BACTERIA AND THEIR PHOSPHATE SOLUBILIZATION

by

ISHANKA UDAYANGI HEMACHANDRA, MS

DISSERTATION

Submitted in partial fulfillment of the requirements

for the degree of Doctor of Philosophy at

The University of Texas-Arlington

May, 2021

Arlington, Texas

Supervising Committee:

Woo-Suk Chang, Supervising Professor

Michael R. Roner

Paul Chippindale

Joseph Boll

Sen Xu

ABSTRACT

EFFECTS OF pH SHOCK AND HEAT STRESS ON THE GROWTH OF NITROGEN-FIXING BACTERIA AND THEIR PHOSPHATE SOLUBILIZATION

ISHANKA UDAYANGI HEMACHANDRA, PhD

The University of Texas - Arlington, 2021

Supervising Professor: Woo-Suk Chang

Rhizobia are well known for their ability to fix atmospheric nitrogen (N) into ammonia, so called biological nitrogen fixation (BNF), either as free-living bacteria in soil or as symbiotic partners of legume plants. Particularly, symbiotic nitrogen fixation (SNF) gains much attention due to its compatibility as a source of N fertilizer in sustainable agriculture. Symbiotic association between *Bradyrhizobium japonicum* and soybean (*Glycine max*) has been used not only as a model system to study SNF, but also as a means of biological soil fertilization to improve crop yield. However, failure to perform optimal BNF in the field is the major limitation associated with rhizobial inoculants. This is mainly due to the sudden death of bacterial cells caused by abiotic stressors upon introduction into soil. Heat stress and soil acidity are the main causes of death of the inoculants. Therefore, gene expression studies were carried out using microarray technology to identify important genes that are responsible for the survival of the bacterium under heat stress and acid shock. Results revealed that a number of small heat shock proteins are induced under heat stress, presumably to protect cells from accumulation of denatured proteins, while cellular mechanisms such as mobility and cell division are shut off, presumably to save energy to overcome the stressful condition. A similar response was triggered under acid shock to reserve cellular

energy to fight against the low pH, while several multi-drug resistance efflux pumps played a key role in maintaining neutral pH within cells. Mutagenesis studies proved the importance of multi-drug resistance efflux pump coding gene *blr7593* under acid shock. There were several small heat shock proteins that were induced under both heat stress and acid shock. Interestingly, some of the up-regulated genes (i.e., *blr7740*, *blr2203* and *blr2694*) are not vital for the survival of the bacterium under the stressful conditions. The second objective of the study was to investigate the possibility of employing *B. japonicum* as a phosphate biofertilizer. Studies showed that *B. japonicum* is capable of solubilizing inorganic phosphate. It was found that *B. japonicum* produces pyrroloquinoline quinone (PQQ) which is the prosthetic group of glucose dehydrogenase (GDH) which may have an indirect effect on phosphate solubilizing. The findings of this work encourage the use of a biofertilizer with dual functions of BNF and phosphate solubilization to create a green economy by mitigating the adverse effects of synthetic fertilizers on environments and human health.

ACKNOWLEDGEMENTS

First and foremost, my heartiest gratitude goes to the most amazing Principal Investigator, I ever met in my academic career Dr. Woo-Suk Chang for his endless support and encouragement throughout my journey. This work would have not been successful without his guidance and advice. As my academic supervisor he has taught me all the necessary techniques required to acquire skills and knowledge in the field of microbial genetics to become a leading researcher.

I would also like to thank the other members of my committee, Drs. Michael R. Roner, Paul Chippindale, Joseph Boll, and Sen Xu for providing their expertise and knowledge into my research work.

I am thankful for those who have helped and collaborated on my projects including all the current/ previous graduate and undergraduate students of Chang lab.

Finally, I would like to thank the Department of Biology at the University of Texas – Arlington including all the staff members who helped and assisted me in many ways during my academic career to fulfill my goal. Thank you very much for making me feel like UTA as home away from home.

April 15, 2021

DEDICATION

I would like to dedicate this work to my beloved husband, Dr. Sampath B. Alahakoon, who is the strength of my life. He pushed me towards the success being my buffer during all the ups and downs in this journey. I would not be here without his support. I would also like to dedicate this work to my parents, Mr. P.A. Hemachandra and Mrs. D.M. K. Jayamenike, Mr. A.M.S.R. Alahakoon and Mrs. A.P.L.C. Alahakoon, who gave me this beautiful life and all the power to face challenges in life to reach my goal. I should not forget my brothers P.A.S.M. Hemachandra, P.A.V.V. Hemachandra, Dilan Karunathilake and sisters A.M.S.J. Alahakoon, Hasitha Asanthi for cheering me up until I complete my work. Finally, this work would be dedicated to everyone who helped in many ways to reach my academic goals.

LIST OF FIGURES

Figure 2- 1. An illustration showing the three regulatory mechanisms of heat shock gene response in *B. japonicum*. 48
Figure 2- 2. Functional categories of statistically significant, differentially expressed genes under heat stress condition at 42°C for 30 min.. 67
Figure 2- 3. Growth comparison of the wild type *B. japonicum* USDA110 under heat stress.... 68
Figure 2- 4. Survivability% of the wild type *B. japonicum* USDA110 under heat stress........... 69
Figure 2- 5. Growth comparison of the wild type *B. japonicum* USDA110 and mutant *blr7740* under normal conditions.. 70
Figure 2- 6. Growth comparison of the wild type *B. japonicum* USDA110 and mutant *blr7740* under normal conditions.. 71
Figure 2- 7. Growth comparison of the wild type *B. japonicum* USDA110 and mutant *blr2203* under normal conditions.. 72
Figure 2- 8. Growth comparison of the wild type *B. japonicum* USDA110 and mutant *blr2203* under normal conditions.. 73
Figure 2- 9. Survivability% of the wild type *B. japonicum* USDA110, mutant *blr7740* and mutant *blr2203* under heat stress.. 74

Figure 3-1. Changes of pH in 100 ml AG culture media (pH 6.8) upon addition of 500 µl of HCl with different concentrations over time.. 109
Figure 3-2. Growth of *B. japonicum* USDA110 in 100 ml of AG media (pH 6.8) upon exposure to acid shock by addition of 500 µl of HCl with different concentrations over time.. 110
Figure 3-3. Growth of *B. japonicum* USDA110 in 100 ml of AG media (pH 6.8) upon exposure to acid shock by addition of 500 µl of 2N HCl and 3N HCl over time.. 111
Figure 3-4. Growth of *B. japonicum* USDA110 in 100 ml of AG media (pH 6.8) upon exposure to acid shock by addition of 500 µl of 2N HCl and 3N HCl over time.. 112
Figure 3-5. Correlation between microarray and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) data of *B. japonicum* USDA110 for 15 genes.. 113
Figure 3-6. Functional categories of statistically significant, differentially expressed genes under acid shock at 500 µl of 2N HCl in 100 ml of AG media for 4h.. 114
Figure 3-7. Growth comparison of the *blr2694* mutant and the wild type *B. japonicum* USDA110 in AG medium (pH 6.8) at 30°C.. 115
Figure 3-8. Growth comparison of the *blr2694* mutant and the wild type *B. japonicum* USDA110 in AG medium (pH 6.8) at 30°C.. 116
Figure 3-9. Growth comparison of the *blr7593* mutant and the wild type *B. japonicum* USDA110 in AG medium (pH 6.8) at 30°C.. 117
Figure 3-10. Growth comparison of the *blr7593* mutant and the wild type *B. japonicum* USDA110 in AG medium (pH 6.8) at 30°C.. 118
Figure 3-11. Survivability comparison of wild type, *blr2694* & *blr7593* mutants in response to acid shock by exposing the cultures to 500 µl of 2N HCl in 100 ml of AG media for 4 h. 119

Figure 4-1. Growth of *B. japonicum* USDA110 in Bergesen's minimal medium (BMM) supplemented with 0.4% glycerol as sole carbon source with different PQQ concentrations.. . 147
Figure 4-2. Growth of *B. japonicum* USDA110 in Bergesen's minimal medium (BMM) supplemented with 0.4% glycerol as sole carbon source with dissolved phosphorous and without dissolved phosphorous; tri calcium phosphate added as sole source of phosphate.. 148
Figure 4-3. pH changes and solubilized phosphorus concentration in *B. japonicum* USDA110 culture in Bergesen's minimal medium (BMM) supplemented with 0.4% glycerol as sole carbon source with tri calcium phosphate as sole source of phosphorous.. 149
Figure 4-4. Comparison of the activity of reconstituted holo-GDH enzyme with culture supernatant of *B. japonicum* USDA110 from different growth conditions.. 150
Figure 4-5. Comparison of PQQ concentration in *B. japonicum* USDA110 culture supernatants grown under different conditions.. 151

LIST OF TABLES

Table 1-1. A summary of Phosphate solubilizing microorganisms (PSMs) and their diversity.. 25
Table 1-2. A summary of different organic acids produced by Phosphate solubilizing microorganisms (PSMs).. 27

Table 2-1. List of bacterial strains and plasmids... 62
Table 2-2. Functional roles of significant genes responding to heat stress................................. 64
Table 2-3. Significantly up-regulated genes in response to heat shock at 42°C for 30 min. 65
Table 2-4. Significantly down-regulated genes in response to heat shock at 42°C for 30 min. .. 66

Table 3- 1. List of bacterial strains and plasmids.. 101
Table 3-2. List of candidate genes selected for qPCR from *B. japonicum* USDA110 microarray study.. 103
Table 3-3. List of primers used for qPCR genes... 104
Table 3-4. Functional roles of significant genes responding to acid shock. 106
Table 3-5. Significantly up-regulated genes in response to acid shock with 2NHCl for 4 h..... 107
Table 3-6. Significantly down-regulated genes in response to acid shock with 2NHCl for 4 h.108

Table 4-1. List of genes up regulated in response to exogenous PQQ (10 µM)........................ 145
Table 4-2. List of genes down regulated in response to exogenous PQQ (10 µM).................. 146

Table A- 1. Old and new gene IDs of up and down regulated genes in response to heat shock.161
Table A- 2. Old and new gene IDs of up and down regulated genes in response to acid shock.163
Table A- 3. Old and new gene IDs of up and down regulated genes in response to 10 µM PQQ. 166

TABLE OF CONTENTS

ABSTRACT i
ACKNOWLEDGEMENTS iii
DEDICATION iv
LIST OF FIGURES v
LIST OF TABLES vii
CHAPTER 1 1
INTRODUCTION 1
Importance of biological nitrogen fixation (BNF) and rhizobia 1
Symbiotic association between soybean and bradyrhizobia 3
Rhizobial inoculants and constraints 6
Major constraints in agricultural fields and stress responses of rhizobia 9
Effects of heat stress on rhizobia and SNF 10
Effects of soil acidity on rhizobia and SNF 12
Effects of phosphate on SNF 14
Phosphorous (P) limitation and phosphate solubilization in soil 15
Phosphorous solubilizing microorganisms (PSMs) 17
Mechanisms of inorganic phosphate solubilization 19
Importance of pyrroloquinoline quinone (PQQ) and its role in mineral solubilization 22
CHAPTER 2 42
PHYSIOLOGICAL RESPONSES OF THE SOYBEAN SYMBIONT *BRADYRHIZOBIUM JAPONICUM* TO HEAT STRESS 42
ABSTRACT 42
INTRODUCTION 43
MATERIALS AND METHODS 48
RESULTS 53
DISCUSSION: 56
CHAPTER 3 82
PHYSIOLOGICAL RESPONSES OF THE SOYBEAN SYMBIONT *BRADYRHIZOBIUM JAPONICUM* TO ACID SHOCK 82
ABSTRACT 82
INTRODUCTION 83
MATERIALS AND METHODS 87

RESULTS 92
DISCUSSION 95
CHAPTER 4 127
DEVELOPMENT OF RHIZOBIAL INOCULANTS WITH DUAL FUNCTION: NITROGEN FIXATION AND PHOSPHATE SOLUBILIZATION 127
ABSTRACT 127
INTRODUCTION 128
MATERIALS AND METHODS 132
RESULTS 138
DISCUSSION 141
.... 148
CHAPTER 5 157
CONCLUSIONS 157
APPENDIX 161

CHAPTER 1

INTRODUCTION

Importance of biological nitrogen fixation (BNF) and rhizobia

Nitrogen (N) is one of the key elements of life on the earth. It is a constituent of proteins and other vital biological macromolecules such as nucleic acids which form the basis of life (Shridhar et al., 2012). Humans and animals need N in the form of proteins while plants need it in the form of ammonium ions. Atmosphere is the largest reservoir of N which contains 78% of molecular dinitrogen (N_2) by its volume. However, this huge amount of N_2 is not bioavailable. Unfortunately, neither plant nor animal species can reduce dinitrogen into ammonia and use directly. The N cycle involves in transformation of atmospheric dinitrogen into readily usable forms such as ammonia in several ways. Lightning is one way of fixing N naturally (Erisman et al., 2011). Another way is industrial nitrogen fixation. The most well-known industrial means is the Haber-Bosch process which directly synthesizes ammonia from nitrogen and hydrogen and thus produces synthetic N fertilizer. Immediate crop responses upon its application, increased crop production, and convenience of handling have made synthetic N fertilizers the popular means of improving and restoring soil fertility of agricultural lands. Biological nitrogen fixation (BNF) is the other way accomplished only by prokaryotes. Prior to industrial revolution, BNF was the main supply of N to plants and animals. It is the process of converting atmospheric dinitrogen into ammonia catalyzed by nitrogenase enzyme in the living microorganisms. BNF is the cheapest way of fixing nitrogen since the breaking down of the triple bond between two nitrogen atoms is highly energetically costly (Galloway et al., 2004).

Substantial increase of food supply on growing world population has been accomplished by the introduction of high yielding, improved crop varieties which entail heavy inputs of fertilizers. Excessive use of synthetic N fertilizers has created numerous adverse effects on environments and human health. Considerable portion of N fertilizers applied to soil is lost via leaching into ground water, through rainwater runoff to surface water bodies and through denitrification into atmosphere, causing environmental pollution (Mulvaney et al., 2009). Contamination of ground water bodies which provide drinking water, with nitrate ions is the main causative agent of the fatal disease, blue baby syndrome in infants (Knobeloch et al., 2000; Majumdar, 2003). Washed off nitrates with runoff water into surface water bodies create sever environmental issues through eutrophication which destroys the aesthetic value of the environment making it inhospitable. Eutrophication is the process of creating hypoxic or anoxic conditions in aquatic ecosystems which leads to massive fish kills due to occurrence of algal blooms because of over enrichment with nutrients such as nitrogen and phosphorous. Nitrogen ended up in surface water ways due to agricultural use is the major source of eutrophication (Carpenter, 2005; Rabalais et al., 2007). Nitrogen driven coastal eutrophication is another widespread environmental catastrophe which causes loss of biodiversity, degradation and loss of coral reefs and alteration of oceanic food webs (Cai et al., 2011; Howarth, 2008; Scavia & Bricker, 2006).

Therefore, BNF is of significance as an efficient and effective source of reactive nitrogen in agriculture and as a long-term strategic plan to overcome negative effects of synthetic N fertilizers. BNF can be carried out only by some prokaryotes, belonging to two domains Bacteria and Archaea. Nitrogen-fixing ability is widespread in different taxa of the Bacteria, including Proteobacteria, Cyanobacteria, Clostridia, Chlorobi, and Actinobacetria. These bacteria possess nitrogenase and can convert atmospheric N_2 into ammonia using their biological machinery.

Therefore, they are termed as biological nitrogen fixers and considered as a key component in the global nitrogen cycle (Shridhar et al., 2012; Vitousek et al., 2002). Nitrogen fixers can be either free-living, or symbiotic microorganisms (Khosro & Yousef, 2012). Free-living nitrogen fixers are living as soil saprophytes and fix nitrogen independently from the rhizosphere or plant exudates. On the other hand, the genera of *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium*, and *Sinorhizobium* collectively known as rhizobia induce nodules on roots of legume plants and occasionally on stems and live inside the nodules (Khosro & Yousef, 2012). They differentiate into specialized forms called bacteroids inside the nodules and provide the host plant with ammonia under microoxic conditions. In this symbiotic relationship, both partners are benefitted since the host plant supply organic compounds to bacteroids in return. Among the above-mentioned different forms of BNF, symbiotic nitrogen fixation is the most advantageous and efficient method since the host plant gets a personalized source of nitrogen from which it can directly assimilate ammonia. Consequently, symbiotic nitrogen fixation becomes ecologically and economically pivotal as a sustainable way of enhancing crop productivity as well as eliminating detrimental effects of synthetic N fertilizers in ecosystems.

Symbiotic association between soybean and bradyrhizobia

Soybean (*Glycine max* (L.)) is the most grown legume crop in the world, occupying 50% of the world's legume crops growing area. The United States, Brazil, Argentina, China and India are the topmost countries that are involved in soybean cultivation in large scale (Chang et al., 2015). Soybean is originated in China and ever since it is popular as a source of plant protein, dietary fibers, starch, lipids, and minerals in both human and livestock diets (Li et al., 2008). Additionally, soybean has a medicinal importance as a source of antioxidants (Omoni & Aluko, 2005), which are beneficial for human health due to high content of phenolic compounds such as

phenolic acids and flavonoids (Couto et al., 2011; Dabrowsk & Sosulski, 1984). Moreover, it has been found that soybean has high levels of isoflavones, a class of phytoestrogens that might fight against cardiovascular diseases and cancers (Mazur et al., 1998). Furthermore, soybean has numerous health benefits for patients who are suffering from high cholesterol levels and diabetics (Silva et al., 2013). Not only is soybean important in human dietary, but also it is famed for an eco-friendly input coming from renewable biomass for biofuel industry due to its high seed oil content. Soybean derived biodiesel has noteworthy advantages of reduction in fossil energy usage and greenhouse gas emission more than 52% and 57%, respectively, compared to petroleum fuels (Huo et al., 2011). Currently, in the United States the majority of biodiesel is produced from soybean. Therefore, it is apparent that soybean is one of the topmost economical crops that are cultivated globally.

Besides, soybean plants are biologically and ecologically significant due to their vital role in global nitrogen cycle and sustainable agriculture via its contribution in nitrogen fixation. Symbiotic nitrogen fixation (SNF) is an important characteristic of most of the members in the family *Fabaceae* (legumes). As a legume crop, soybean establishes the nitrogen fixing symbiosis with several groups of rhizobia, predominantly genus *Bradyrhizobium. Bradyhrizobium japonicum* is the prominent symbiotic partner who improves growth and yield by providing the soybean plants with fixed atmospheric nitrogen. Hence, the soybean-*B. japonicum* symbiosis has been considered as a model to study plant-rhizobium interactions over decades. Soybean contributes remarkably towards the supply of reactive nitrogen species to biosphere through SNF with 16.4Tg of fixed nitrogen which represents 77% of the total N fixed by legume crops annually (Herridge et al., 2008). Moreover, SNF is highlighted because of its ability to satisfy about 50-

60% of the nitrogen requirement of soybean as a cheaper alternative for synthetic N fertilizer (Herridge et al., 2008).

Establishment and development of the symbiosis is a result of a complicated series of chemical signaling between legumes and rhizobia which is highly selective. Not every rhizobium is compatible with every legume since this is a host specific relationship where the appropriate partners should come together to form a successful symbiosis (Stougaard, 2000). Each step in the process is crucial and requires its optimum condition to accomplish the goal of effective SNF. A sequence of morphological changes in the cells of both partners is evoked upon recognition of each other through chemical signal exchange. Symbiosis between soybean and *B. japonicum* is initiated with sensing and responding to root exudates in the rhizosphere. Genistein and daidzein produced through the phenylpropanoid pathway in soybean are the major isoflavones that induce nodulation (*nod*) genes in *B. japonicum.* These compounds are secreted by soybean roots into the rhizosphere and recognized by the bacteria. Particularly genistein is known to trigger all the *nod* genes including the *nodYABCSUIJ* operon (Lang et al., 2008; Lee et al., 2012; Subramanian et al., 2007). Additionally, coumestrol is another inducer of *nod* genes of *B. japonicum* (Kosslak et al., 1987). Symbiosis related genes of *B. japonicum* that are located in its chromosome are responsible for production and secretion of nodulation (Nod) factors (Kaneko et al., 2002). Upon recognition of root exudates, the Nod factors (lipochitooligosaccharide) produced by the bacteria trigger the mechanisms for bacterial invasion and nodulation (D'Haeze & Holsters, 2002; Geurts & Bisseling, 2002). Bacteria are adsorbed on to plant root surfaces causing root hair deformation followed by trapping of the bacteria in between the cell walls. Rhizobia enter the plant roots and move towards the inner cortical cells of the roots through a plant derived infection thread. Then, the bacteria are released into developing nodule primordium surrounded by plant originated membrane called the

peribacteroid membrane (Gage, 2017; Pierre et al., 2013; Whitehead & Day, 1997). Inside the root nodules rhizobia differentiate into their N fixing state called as bacteroids. The peribacteroid membrane enclosed bacteroids is called as a symbiosome in which dinitrogen is converted into ammonia under the oxygen limited environment (Oke & Long, 1999).

SNF is controlled by two major classes of genes: the *nif* genes and *fix* genes. Reduction of dinitrogen into ammonia in the nitrogen fixation process is catalyzed by the prokaryotic enzyme complex nitrogenase which is encoded by *nifDK* and *nifH* genes. The nitrogenase complex consists of two components, a molybdenum-iron protein (MoFe), called component 1 encoded by *nifK* and *nifD* and an iron containing protein (Fe), called component 2 encoded by *nifH.* The *nif* genes are positively controlled by transcriptional activator NifA and a negative regulator NifL. *nif* genes are regulated by oxygen and fixed nitrogen levels. Nitrogenase enzyme complex is highly sensitive to oxygen and reactive oxygen species (ROS). Additionally, *fix* genes are also involved in the SNF process by sensing microoxic conditions and preparing the bacteroids for microaerobic respiration and dinitrogen reduction (Berman-Frank et al., 2003; Hungria et al., 2005). Once dinitrogen is reduced to ammonia, *B. japonicum* bacteroids assimilate it with the help of glutamate dehydrogenase or through glutamine synthetase/glutamate synthase pathway so that ammonia is incorporated into plant amino acids (Brown & Dilworth, 1975; Franck et al., 2015; Vairinhos et al., 1983).

Rhizobial inoculants and constraints

Incorporation of N into soil through SNF in legume-based cropping systems is a cost-effective method to improve crop yield and production, while mitigating the negative impact of synthetic N fertilizers on human health and environments (Ronner et al., 2016). Generally, natural soils are rich of free-living, associative, and symbiotic nitrogen fixers. However, due to the host

specificity of SNF, presence of the compatible rhizobial strains in the soil is essential. Soybean was originated in Asia and was first introduced to North America (1765) and South America (188-1882). Therefore, in the beginning of this introduction period SNF was probably low due to the absence of compatible rhizobial strains in the soil in North and South America (Chang et al., 2015). Introduction of rhizobial inoculants is critical to ensure effective SNF, especially when soybean is introduced to a new geographic area. As an alternative for low SNF due to the host specificity, several attempts have been made in different geographical regions to introduce genetically engineered soybean varieties that can freely nodulate with indigenous rhizobial strains (Abaidoo et al., 2007; Mpepereki et al., 2000). However, most of the trials with these promiscuous soybean plants were reported unsuccessful to meet the total N requirement in several locations (Sanginga et al., 1996). Therefore, a demand for a better strategy to ensure SNF between soybeans and inoculants has been increasing.

Rhizobium/Bradyrhizobium inoculants have been used over several decades in soybean cultivation. Most of the applications were successfully able to increase the soybean yield as expected. It is common to apply rhizobia inoculants with all the soybean varieties in Brazil and Argentina (Brockwell & Bottomley, 1995; Hungria et al., 2005; Peoples et al., 2009). It has been reported that the laboratory findings on inoculant performance do not often reflect actual field conditions. The fidelity of the commercial inoculants in the field is controversial to some extent. Sudden decline of population size upon application, less survivability in the field in off seasons, and inefficiency in nodulation and SNF are the current issues associated with the inoculant performance. Efficiency of occupying nodules and nitrogen fixation by rhizobia depends on several factors. Survivability and adaptability to different environmental conditions, compatibility with different hosts, and ability in nodulation vary among the strains. In addition, other factors

such as, formulation, storage, transportation, and field application technology of inoculants can be considered to obtain promising results by inoculants. Therefore, collaborating efforts for selection of proper strains, appropriate processing and formulation techniques, proper storage, and correct methods of application are crucial to harness the benefits of SNF. understanding genetics and physiology of the rhizobia is another critical factor that needs to be addressed. Failure to maintain effective population size required for competitive, effective nodulation, and N fixation after field application is the major problem that leads to poor performance by inoculants. Soybean growers who apply the rhizobia in the field expect successful inoculation and subsequent yield improvements. If the inoculants are not doing well in the field, growers are likely going back to the use of synthetic fertilizers. Therefore, globally accepted rules and regulations for legume inoculant formulation and usage should be imposed to enhance the use of the inoculants. In order to accomplish the target, major constraints that hinder the survival and performance of rhizobia in the field should be identified and need to be properly addressed at the context (Herrmann & Lesueur, 2013; Hungria et al., 2005; Thuita et al., 2012). Quality of the inoculants is manageable under highly monitored conditions while the innate capacity of the bacteria is not well known. Hence, the most vital step in formulation of inoculants is the strain selection. The candidate rhizobia should be compatible with the host and genetically and physiologically strong enough to withstand the challenging environmental fluctuation. Because, in the field they are exposed to several biotic and abiotic stress conditions which are often beyond control. Competition induced by ineffective local rhizobia for resources in the rhizosphere as well as for nodule occupancy; potentially can lower the capacity of inoculants to enter the symbiosis stage. In addition, the survival and persistence of rhizobia in soils are mainly restricted by the environmental factors such as extreme temperatures, soil pH, salinity/alkalinity, desiccation, nutrient deficiencies, toxicities

(Hungria & Vargas, 2000; Mabrouk & Omrane, 2012; Zahran, 1999), and predation by protozoa (Danso et al., 1975). Among them, high soil temperature and acidity could cause the most seriously detrimental effects on both rhizobial survival and the nodulation process. Therefore, understanding genetics and physiology of *B. japonicum* under these stressful conditions is important for future manipulation of the bacteria in formulation of robust inoculants with improved performance to maximize BNF.

Major constraints in agricultural fields and stress responses of rhizobia

Use of rhizobial inoculants as an alternative for synthetic nitrogen fertilizers in legume based agricultural systems is an economically and environmentally sustainable approach to improve crop production. Nodule dwelling bacteria and symbiotic bacteroids are released into the environment and remain in soil as free-living heterotrophic saprophytes until they encounter a suitable legume host. Survival and persistence of these free-living bacteria largely depends on soil environmental factors such as soil water content, nutrients, soil pH and temperature. The ability to withstand these conditions is more important for the free-living stage than during symbiosis since the nodule provides a protective atmosphere. Adaptations and persistence in soil during the off seasons as saprophytes is also critical for entering into symbiosis in the next cropping cycle. Moreover, sudden death of applied rhizobia via seed coating or soil application is the other major impediment in developing an enduring inoculum in the field. Population size often rapidly declines soon after introduction to natural soils due to multifarious factors. Any environmental factor that influences the growth and survival of rhizobia have a significant impact on their ability to perform SNF.

Desiccation stress has been identified as one of the major causes of rhizobial death on seeds. A multitude of studies related to desiccation stress responses of rhizobia and genetics of

desiccation tolerance have been reported. Soil pH and temperature also have a remarkable influence on survival and persistence of rhizobia in soil. Effects of soil pH on rhizobial performances have been extensively studied due to worldwide soil acidity (Hungria et al., 2005). Soil acidification is accelerated by the anthropogenic agricultural practices such as growing high yielding crops, continuous removal of harvest and repeated application of synthetic ammonium-based fertilizer and elemental sulfur fertilizers. Specially, legume fields become rapidly acidic (Goulding, 2016; Williams, 1980) due to net release of protons to balance the charge caused by excessive uptake of cations due to formation of NH_4^+ in N fixation. Therefore, it is vital to understand how rhizobia behave in acidic soils to develop an effective biofertilizer.

High temperature stress (i.e., heat stress) is the other factor that rhizobia encounter daily and seasonally. High temperature associated drought could be more stressful for rhizobial survival and symbiosis. This is a common environmental hardship that the rhizobia are confronted with in semi-arid and arid soils (Abd-All et al., 2014).

Effects of heat stress on rhizobia and SNF

Temperature is one of the major factors that govern the growth and survival of microorganisms including rhizobia. Every organism has a range of temperature in which its growth and physiological processes are optimal. The optimum temperature for most of rhizobia is between 25-30°C. However, rhizobia are often subjected to above or below the optimum temperatures which could be stressful throughout their life as a soil saprophyte and a micro-symbiont (Zhang et al., 1996). Temperatures beyond the threshold level create stressful environments for the microorganisms under which, several protective mechanisms are triggered to overcome the stressful condition. High soil temperature is one of the critical factors which decline rhizobial populations in great numbers after applying into soil. Adaptability to high temperatures is an innate

characteristic of rhizobial strains. Most of soybean associated rhizobial isolates are unable to grow at higher temperatures such as 40-45°C (Chen et al., 2002). Negative effects of high temperatures at the time of inoculation on the population size was evident by the reduction of recovery of the *B. japonicum* inoculum applied to soil at 38°C than 28°C. Four to five percent of the inoculum was recovered from the soil after 24 h of inoculation at 28°C, while it was decreased up to less than 0.2% at 38°C (Brockwell et al., 1987; Montañez et al., 1995).

Moreover, high soil temperatures have detrimental effects on both nodule formation and SNF in soybean which results in poor performance of inoculants (Montañez et al., 1995). High temperatures disrupt the initial steps in the nodulation process by distracting chemical communication between two symbiotic partners. Included are retardation of the root hair curling, premature of infection thread development and failure of nodule formation and symbiosis (Lebrazi & Benbrahim, 2014; Sadowsky, 2006). Also, high soil temperatures are more critical than low temperatures on both free-living and symbiotic rhizobia (Zahran, 1999). Many studies have been performed to produce heat stress resistant soybean varieties. Moreover, there has been studies to explore the capabilities of the microsymbiont to tolerate different stressful conditions. The major drawback of biofertilizers is failure of the inoculum to reproduce their *in-vitro* performances at the field level due to lack of adaptability to the abiotic stressors.

High soil temperatures are also a major constraint for BNF of legume species including soybean, guar, peanut, cowpea, and beans. Soil temperature directly influence the growth and existence of both parties of the symbiosis and nodulation process. The optimum temperature range for the nodulation process varies (Zahran, 1999). For most of the rhizobia partners, preferred temperature fluctuates from 28 to 31°C and many are unable to grow at 37°C. However, rhizobia isolated from hot dry regions show tolerance to high temperatures such as 37°C and 40°C. For

example, cowpea associated rhizobia isolated from hot dry Sahel-savannah of West Africa can grow at 37°C and more than 90% of the strains found in this region can grow well up to 40°C (Osa-Afiana & Alexander, 1982; Sadowsky, 2006).

Effects of soil acidity on rhizobia and SNF

pH is one of the most influential environmental factors that affect the growth and existence of microorganisms. Acidity is a much more common abiotic stress condition than alkalinity that microorganisms experience in their niches. Irrespective of the habitat they occupy, microorganisms encounter acidity at different stages of their growth and proliferation. For example, enteric bacteria such as *Escherichia coli* and *Salmonella* spp. must combat acidic conditions during the process of invading the human intestinal tract. They experience extreme pH conditions as low of 1.5-2.5 to less acidic environments of pH 4.0-6.0 from stomach to the intestines (Lin et al., 1996; Xu et al., 2020). *Staphylococcus aureus*, one of the major human pathogens as well as a food poisoning bacterium, is equipped with a multitude of defense mechanisms against sudden acid shocks (Cotter & Hill, 2003). The most adapted method is pumping protons out of the cell to maintain the internal pH at the desired level. Another response is increasing the concentration of alkaline compounds within the cell. Additionally, energy metabolism and metabolic pathways are altered to provide more energy to accelerate defense mechanisms than growth and development (Bore et al., 2007). Bacterial responses to acidic conditions have been widely studied using many model organisms. Studies have shown that both *E. coli* and *Salmonella typhimurium* show an inducible response to the acid shock which is called as adaptive-tolerance response (ATR) (Foster & Hall, 1990) and acid habituation which is ecologically important for the persistence of the microorganisms in an acidic environment. Briefly, these organisms show significantly more resistance to extreme pH shock conditions when they are

cultured at a mildly acidic pH than growing at neutral pH (Foster & Hall, 1990; Goodson & Rowbury, 1989a, 1989b). This system appears to be involved in responding to many other environmental stresses as well. For instance, *E. coli* cells grown in a mildly acidic medium are found to be more resistant to UV light (Goodson & Rowbury, 1991) and acid treated cells of *Listeria monocytogenes* were more heat resistance than cells grown in pH 7.0 (Farber & Pagotto, 1992).

Similarly, sensing and responding to extreme acidic conditions is critical to many soil dwelling bacteria such as plant pathogens and symbionts. Plant pathogens undergo acidic conditions specifically during the invasion of the host plant. To successfully complete the infection process and to colonize the host plant cells, they need to combat acidity in the host environment. Also, some plant pathogens have evolved specific response systems to low pH to induce the genes that are responsible for virulence. These types of microorganisms may possess more complex and interconnected cellular network of detecting and reacting to low pH. For example, tumor causing plant pathogen *Agrobacterium tumefaciens,* which transfers oncogenic DNA into host plants, uses pH as an indicator of the presence of the host. Low pH is one of the factors that induce the expression of virulence (*vir*) genes that facilitate transfer of DNA into host cells. The VirA/ VirG two-component system of *A. tumefaciens* regulates the production of the transferred oncogenic DNA and the DNA transfer machinery from the tumor inducing Ti plasmid in response to low pH (Binns, 2002; Tzfira & Citovsky, 2002). VirA, membranal histidine sensor kinase is autophosphorylated upon exposure to host indicators including low pH and transfers the phosphoryl group to VirG. Phosphorylated VirG activates the expression of the *vir* regulon. This system can be considered as a modified version of responding to low pH since it evokes the virulence of the bacterium instead of housekeeping cellular responses to overcome the acidity.

In addition to the cellular response to low pH, acidity related soil nutrient deficiency is another major barrier for crop growth in agriculture. Phosphate solubilization is an example. Certain level of acidity is created and maintained by the microbial activities to solubilize inorganic phosphate. Bacterial phosphate solubilization generally associated with acidity and ultimately benefit both legume host and symbiotic bacteria by ensuring a continuous supply of soil available phosphate.

Effects of phosphate on SNF

While being essential for plant growth and development, phosphates play a key role in SNF in legumes. Phosphate deficiency directly interrupts SNF by limiting growth and persistence of rhizobia, nodule formation and host plant growth (Isidra-Arellano et al., 2018). In addition, reduced nodule mass due to soil phosphate scarcity has also been reported (Drevon & Hartwig, 1997). However, poor nodule formation and functioning induced by phosphate deficiency is dependent on the innate properties of the host plant and the severity of the phosphate starvation (Kousa et al., 2005). Furthermore, some studies have documented that the both phosphate-efficient and inefficient common bean varieties demand relatively low phosphate concentrations for the optimal nodule initiation. However, further development and functioning of established nodules at later stages is found to be weakened or inhibited by the phosphate starvation (Valverde et al., 2002). In one study that compared the requirement of phosphate for initial nodule formation and the later growth and functioning of the nodulated root system of common beans, nodule numbers and biomass and SNF were significantly reduced due to low availability of usable phosphate. (Hernández et al., 2009). Besides, it has been reported that phosphate deficiency impairs the rhizobial nitrogenase enzyme activity by increasing the nodule permeability to oxygen, resulting in inactivation of the enzyme (Isidra-Arellano et al., 2018). In contrast, Kousa et al. reported that

biomass, size, and efficiency of SNF of an individual nodule were independent of phosphate availability (Kousa et al., 2005). Hence the relationship between phosphate demand of host legumes and the rhizobial partner for effective symbiosis is complicated and controversial. Therefore, adequate reserves of available soil phosphorous (P) or the phosphate solubilization by rhizobia is specifically vital to ensure SNF between legumes and their partners.

Phosphorous (P) limitation and phosphate solubilization in soil

Phosphorous (P) is one of the most essential plant nutrients which has a structural and functional importance in growth and development. It is a major component of nucleic acids, enzymes, co-enzymes, phospholipids, and accounts for between 0.2-0.8% of plant dry weight (Sharma et al., 2013). It also plays an important role in major metabolic pathways and physiological processes in plants including photosynthesis, energy production, storage and transportation, signal transduction, macromolecular biosynthesis, respiration, growth and development of roots, strengthening the stalk and stems, formation of flowers and seeds, crop maturity and quality of crops, cell division and enlargement, resistance to plant diseases, transformation of sugar to starch and transmission of the genetic information (Kalayu, 2019; Khan et al., 2009; Khan et al., 2010). Interestingly, P is also involved in BNF in legumes (Kousa et al., 2005). P deficiency retards plant growth and ultimately reduces the crop yield. Unlike N, there is no large reservoir of atmospheric P which can be made biologically available for plant uptake. Plants absorb P from the soil solution either in the form of HPO_4^{2-} or $H_2PO_4^-$ anions based on soil pH. Although P is abundant in most of agricultural soils in both inorganic and organic forms, its bioavailability in the soil solution is limited because of P fixation which is the removal of available phosphate from the soil solution into the solid phase (Filippelli, 2008). Availability of P in soil is greatly affected by soil pH and P fixation. Traditionally, P deficiency in soil is overcome by adding

synthetic phosphate fertilizers. However, only a small amount of added phosphate fertilizers is absorbed up by plant roots and a larger portion (around 75-90%) is precipitated with metal cation complexes in soil and become unavailable for plants (Kalayu, 2019). Therefore, to fulfill the P requirement of plants, frequent application of phosphate fertilizers is needed. The repeated P fertilization leads to accumulation of inorganic phosphate in soil and creates adverse effects on environments including eutrophication, carbon footprint, and soil fertility depletion by disturbing the microbial community.

Soil is the major source of phosphorous for all microorganisms on the earth. P naturally exists in soils as an insoluble form, either organically or inorganically. Inorganic phosphates contain P bound on to primary minerals. Organic P is derived from decaying plants, animals, and microorganisms. About 95 to 99% of soil P is in the form of insoluble phosphates and the concentrations of soluble P vary depending on soil types. Soil P deposits undergo different transformation and translocation processes through which they become either available to plants or immobile. Weathering, mineralization, and desorption release insoluble phosphorous into the soil solution while immobilization, precipitation and adsorption decrease bioavailable P. Weathering is the natural process through which P containing minerals slowly release phosphorous into soil solution. Mineralization is the microbial conversion of organic P into forms of plant available P in soil. In contrast, immobilization occurs when the bioavailable P is consumed by soil microorganisms. In this process, plant available orthophosphates are converted into organic P in the microbial system and taken away from the soil solution. This organic P will become available when these microorganisms are dead. Adsorption is the temporary binding of bioavailable P on to soil particles which will be released to soil solution later through desorption. P also become unavailable due to precipitation with the dissolved metal ions in the soil solution. Unlike

adsorption, precipitation is a more permanent reaction in which chemical properties of phosphate are changed. Both precipitation and adsorption are pH dependent. Bioavailable P is precipitated with Ca^{2+} in alkaline soils and with Al^{3+} and Fe^{3+} in acidic soils. Precipitated and absorbed P is washed away with runoff water and end up in sediments. Dissolved P is leaching down through vertical water movements leaving the plant accessible soil solution. Consequently, a promising approach which could supply adequate P to plants is still required in sustainable agriculture. Like nitrogen fixing bacteria renowned as N-biofertilizer in agriculture for decades. Phosphorous solubilizing bacteria are also being used as biofertilizer since 1970s. However, it needs to be improved as inoculants to replenish bioavailable P in soil solution. Hence, it is imperative to investigate the involvement of soil bacteria in mobilization of P among different pools of soil phosphates. Microorganisms mediated P management would be an eco-friendly and cost-effective alternative for chemical phosphate fertilizers in sustainable agriculture.

Phosphorous solubilizing microorganisms (PSMs)

Soil microorganisms play a significant role in global nutrient circulation including both P and N cycles. They are the key transformers of nutrient reserves to available forms for life on the earth. A vast variety of soil microorganisms including bacteria, fungi, actinomycetes and algae possess P solubilization and mineralization ability which makes them possible candidates as a P biofertilizer (Table 1-1). Phosphate solubilizers are ubiquitous in nature and have much more benefits on crop plants and microflora and fauna in agricultural ecosystems, but varying in occurrence, density, and phosphate solubilization ability from one system to another. Their diversity is depending on the biotic and abiotic factors of the soil (Srinivasan et al., 2012) *Pseudomonas* spp.*,* *Agrobacterium* spp. and *Bacillus circulans* are well studied soil bacteria with P mobilization via solubilization and mineralization (Babalola & Glick, 2012). The greatest

number of phosphates solubilizers is reported in genus *Bacillus* followed by *Pseudomonas*. Additionally, a number of strains of *Azotobacter, Bacillus, Burkholderia, Enerobacter, Erwinia, Kushneria* (Zhu et al., 2011)*, Paenibacillus, Ralstonia, Rhizobium, Rhodococcus, Serratia, Bradyrhizobium, Salmonella, Sinomonas* and *Thiobacillus* (Sharma et al., 2013; Srinivasan et al., 2012) are known to be involved in P solubilization and mineralization. Among the phosphate solubilizing fungi, *Aspergillus* and *Penicillium* are most common followed by many other genera such as *Achrothcium, Alternaria, Arthrobotrys, Aspergillus, Cephalosporium, Cladosporium, Curvularia, Cunninghamella, Chaetomium, Fusarium, Glomus, Helminthosporium, Micromonospora, Mortierella, Myrothecium, Oidiodendron, Paecilomyces, Penicillium, Phoma, Pichia fermentans, Populospora, Pythium, Rhizoctonia, Rhizopus, Saccharomyces, Schizosaccharomyces, Schwanniomyces, Sclerotium, Torula, Trichoderma, and Yarrowia* (Mogk et al., 2003). Additionally, actinomycetes and algae such as cyanobacteria and mycorrhiza as another group of plant growth promoters have also exhibited the potential P solubilizing properties. Studies have shown that approximately 20% of actinomycetes possess the ability to solubilize P including the common genera *Streptomyces* and *Micromonospora*. Arbuscular mycorrhizal fungi (AMF) are also known to enhance plant growth when co-inoculated with interactive rhizobacteria (Sashidhar & Podile, 2010)

Furthermore, symbiotic nitrogen fixing bacteria have also been reported to possess P solubilizing ability (Zaidi et al. 2009). For example, *Rhizobium leguminosarum bv. Trifolii* and *Rhizobium* species nodulating *Crotalaria* species (Sashidhar & Podile, 2010) were identified as inorganic and organic P solubilizers. *R. meliloti* SU47 (Halder & Chakrabartty, 1993), *R. leguminosarium by. viceae* strain TAL 1236 and 1402 and *Bradyrhizobium* strains (Abd-Alla, 1994; Antoun et al., 1998) were found to solubilize phosphate from hydroxyapatite.

Mechanisms of inorganic phosphate solubilization

There are three major components such as i) dissolution-precipitation, ii) adsorption-desorption and iii) mineralization-immobilization in the soil P cycle. Phosphate solubilizing bacteria deploy a variety of methods to make insoluble inorganic phosphates available for plants. The principal mechanism is the production of mineral dissolving compounds such as organic acids, siderophores, protons, hydroxyl ions and CO_2 which cause lowering of soil pH. Additionally, they are responsible of dissolving inorganic phosphates through chelation, and mineralization.

Organic acid production:

Dissolving mineral bound phosphorous through lowering soil pH is the most prominent mechanism employed by phosphorous solubilizing bacteria (PSB). In alkaline soils phosphate is found as calcium phosphate which is an unavailable precipitate of phosphate for plants. Their solubility is accelerated by decline of soil pH. PSB achieve this by production of organic acids or the release of protons. (Kumar et al., 2018; Walpola & Yoon, 2012). Organic acids are mostly produced during the metabolism of organic carbon sources by bacteria via direct oxidation or fermentation in the periplasmic space. The PSBs release different types of organic acids (Table 1-2) in various amounts and phosphate solubilization is largely dependent on type and strength of the acid. Organic acids that primarily involve in phosphate solubilization are acetic, formic (monocarboxylic acids); lactic, gluconic, glycolic (monocarboxylic hydroxy acids); 2-ketogluconic (monocarboxylic keto acids); oxalic, succinic (dicarboxylic acids); malic (dicarboxylic hydroxy acids); citric (tricarboxylic hydroxy acids), fumaric, , tartaric, malonic, glutaric, propionic, butyric, glyoxalic, and adipic acid (Hemida et al., 2014; Kumar et al., 2018; Selvi et al., 2017; Walpola & Yoon, 2012; Yousefi et al., 2011). Gluconic acid and 2-ketogluconic acid are the ubiquitous organic acids among the phosphate solubilizing bacteria. Gluconic acid is

the key organic acid produced by PSBs such as *Pseudomonas* sp., *Erwinia herbicola*, *Burkholderia cepacia* (Rodríguez & Fraga, 1999) while 2-ketogluconic acid is found in *Rhizobium leguminosarum*, *Sinorhizobium meliloti* and *Bacillus firmus*. Additionally, PSMs also create acidity in the surrounding by evolution of CO_2.

Inorganic acid production:

Inorganic acids produced by nitrifying and sulfur oxidizing bacteria are also involved in dissolving inorganic phosphates. They are produced during bacterial oxidation of nitrogenous or inorganic sulfur compounds. Bacteria derived sulfuric acid, nitric acid, and carbonic acids react with insoluble metal complexes of inorganic phosphate and release phosphate ions into the soil solution (Kalayu, 2019; Walpola & Yoon, 2012). However, inorganic acids are reported to be less effective than the organic acids in phosphate solubilization.

Chelation:

Chelation is done by both organic and inorganic acids produced by PSMs. These acids are strong chelators of cations such as calcium, iron, and aluminum. The hydroxyl and carboxyl groups of the acids chelate the cations bound to phosphates and compete with phosphate for adsorption sites. They saturate the active attachment sites on Al and Fe oxides and stabilize them. They are called chelates. This process releases phosphate ions from the insoluble cation complexes into soil solution. Gluconic acid and 2-keto-gluconic acid are the most powerful chelators (Khan et al., 2009; Walpola & Yoon, 2012). In addition, humic acid and fulvic acid which are released by microorganisms during degradation of plant organic matter are also reported to be more powerful chelators which are responsible for inorganic phosphate solubilization.

Proton extrusion:

In some studies, conducted with P solubilizers it was revealed that phosphate solubilization is still possible, even without production of organic acids. For instance, a HPLC analysis of culture supernatant of *Pseudomonas* sp., did not detect any organic acid when phosphate solubilization occurred (Illmer & Schinner, 1995). They proposed that the release of protons associated with respiration or NH_4^+ assimilation as the source of acidity in the medium that is responsible for releasing phosphates.

Exopolysaccharide production (EPS):

Microbial EPS are polymers containing carbohydrates excreted by bacteria and fungi onto the outside of their cell wall. The composition and structure of EPS are highly diverse among bacterial strains and that can be attributed to various functions. A direct relationship between EPS production and phosphate solubilization has not yet been established. However, recently reported study revealed that *Enterobacter sp. (EnHy-401), Arthrobacter sp. (ArHy-505), Azotobacter sp. (AzHy-510)* and *Enterobacter sp. (EnHy-402)* is capable of solubilizing tri calcium phosphate and exhibited increased production of EPS. However further studies should be encouraged to understand the relationship between EPS production and phosphate solubilization (Sharma et al., 2013).

Siderophore production

Siderophores are produced by most of microorganisms under iron deficient conditions and have high affinity to fixed iron that is bound on to metal ions or organic materials. Siderophores dissolve iron containing minerals and organic materials to liberate iron under iron limited environments. It is reported that majority of PSMs release siderophores even though it is not

widespread as a phosphate solubilizing mechanism (Vassilev et al., 2006). However, the function of siderophores ultimately contributes towards making available of P for plant utilization (Sharma et al., 2013).

Importance of pyrroloquinoline quinone (PQQ) and its role in mineral solubilization

Organic acid mediated phosphate solubilization is widespread among PSB and they release different types of organic acids while gluconic acid and 2-ketogluconic acid are ubiquitous. Gluconic acid is the key organic acid produced by PSBs such as *Pseudomonas* sp., *Erwinia herbicola*, *Burkholderia cepacia* (Rodríguez & Fraga, 1999) while 2-ketogluconic acid is found in *Rhizobium leguminosarum*, *Sinorhizobium meliloti* and *Bacillus firmus*. The well-studied mechanism of bacterial phosphate solubilization is through secretion of gluconic acid (Goldstein, 1995) which is produced through direct oxidation of glucose by glucose dehydrogenase (GDH) which requires pyrroloquinoline quinone (PQQ) as the redox prosthetic group (Duine et al., 1979; Naveed, 2016). PQQ, also known as methotaxin is a water soluble, heat stable tricyclic ortho-quinone (Puehringer et al., 2008) functioning as the redox cofactor for various bacterial dehydrogenases such as methanol, ethanol and glucose dehydrogenases and the first to be found in this cofactor family. Structural data from the PQQ biosynthesis show that PQQ is derived from the two amino acids glutamate and tyrosine. Many bacteria possess the genes (*pqqABCDEF/G*) required for the PQQ biosynthesis. Bacterial production of PQQ is the primary source, while neither plant nor animals can synthesis PQQ. Although not synthesized in animals, it functions in the numerous ways, including healing of neurological injury, acting as an anti-melanogenic agent against hyper pigmentation, immunity booster, treatment for insomnia, cancer fighter, liver fibrogenesis, signal transduction via mitochondrial biogenesis, and protecting from cardiac disease. In plants, PQQ involves in phosphate solubilizing activities, plant growth promotion,

antifungal activities, induced systemic resistance. Recently, PQQ has shown multiple applications in modern technology as a bio-electro catalysis, conductive polymeric fibers in polymer technology, and charge transfer mediated nanoparticles. While being the sole source of natural PQQ, microbes got many advantages as a growth stimulant, antibiotics, energy transduction, ATP synthesis during oxidation, intracellular signaling and DNA repair. GDH is a quinoprotein that requires PQQ and possesses binding sites for Ca^{2+}, ubiquinone and for substrate glucose for its activity. Two types of GDH enzymes have been identified, GDH A and GDH B based on its location within the cell. GDH A, a membrane-bound enzyme (m-GDH) is more prominent and reported in *Acinetobacter calcoaceticus, Pseudomonas aeruginosa, Gluconobacter suboxydans, Klebsiella aerogenes Acinetobacter lwoffi and Escherichia coli.* GDH B is soluble (s-GDH) and reported only from *Acinetobacter calcoaceticus* (Jansen et al., 1988). The presence of a PQQ-dependent GDH and inorganic phosphate solubilization capabilities have been reported in rhizobia as well (Abd-Alla, 1994; Halder & Chakrabartty, 1993). However, some rhizobia such as *Rhizobium legumiosarum* and *Sinorhizobium meliloti* and several strains of *Escherichia coli* were able to produce gluconate from glucose in the presence of exogenous PQQ proving that they are unable to synthesize PQQ and possess the apo-GDH. In contrast some bacteria such as *Deinococcus radiodurans* synthesize PQQ but, do not exhibit phosphate solubilization capability (Khairnar et al., 2003). Therefore, PQQ-dependent GDH activity, PQQ biosynthesis and inorganic phosphate solubilization is highly variable and widespread among bacteria and rhizobia.

In this study, the potentiality of improving performances of the well-known N biofertilizer *B. japonicum* was explored along three different aspects, including heat stress, soil acidity, and P solubilization, which should be broadly addressed to achieve the long-term goal of maintaining sustainable agriculture. The aim of this study is to investigate and understand how *B. japonicum*

is tolerating and responding to major abiotic stressors in soil, heat stress and acidity to survive and remain infectious to carry out SNF effectively. Changes in gene expression and physiological processes of the bacterium in response to heat and acid shock will be extensively analyzed using whole genome transcriptome profiling and mutagenesis studies to deduce the importance of significantly regulated genes and operons. Moreover, in this study we plan to excavate the inorganic phosphate solubilizing properties of *B. japonicum* to find the possibility of promoting it as a P biofertilizer through physiological and transcriptomic studies.

Table 1-1. A summary of Phosphate solubilizing microorganisms (PSMs) and their diversity.

PSM	Examples	Reference
Bacteria	*Acetobacter sp.*	(Joseph & Jisha, 2009)
	Acetobacter liquefaciens	(Joseph & Jisha, 2009)
	Acinetobacter sp.	(Rodríguez & Fraga, 1999)
	Aerobacter aerogenes	(Rodríguez & Fraga, 1999)
	Bradyrhizobium japonicum	(Antoun et al., 1998)
	Burkholderia cepacia	(Rodríguez & Fraga, 1999)
	Enterobacter aerogenes	(Chung et al., 2005)
	Enterobacter aerogenes	(Chung et al., 2005)
	Pseudomonas putida	(Cattelan et al., 1999)
	Ralstonia sp.	(Pérez et al., 2007)
	Rhizobium sp.	(Halder & Chakrabartty, 1993)
	Rhizobium meliloti	(Halder & Chakrabartty, 1993)
	Rhizobium leguminosarum biovar Phaseoli	(Chabot et al., 1999)
	Rhizobium loti	(Halder & Chakrabartty, 1993)
	Serratia phosphaticum	(Tilak et al., 2006)
Fungi	*Aspergillus awamori, A. niger, A. tereus, A. flavus, A. nidulans, A. foetidus, A. wentii. Fusarium oxysporum, Alternaria teneius, Achrothcium sp. Penicillium digitatum, P lilacinium, P balaji, P. funicolosum, Cephalosporium sp. Cladosprium sp., Curvularia lunata, Cunnighamella, Candida sp., Chaetomium globosum, Humicola inslens, Humicola lanuginosa, Helminthosporium sp., Paecilomyces fusisporous, Pythium sp., Phoma sp.,*	

	Populospora mytilina, Myrothecium roridum, Morteirella sp., Micromonospora sp., Oideodendron sp., Rhizoctonia solani, Rhizopus sp., Mucor sp., Trichoderma viridae, Torula thermophila, Schwanniomyces occidentalis, Sclerotium rolfsii.	(Sharma et al., 2013)
Actinomycetes	*Actinomyces, Streptomyces.*	(Sharma et al., 2013)
Cyanobacteria	*Anabena* sp., *Calothrix braunii, Nostoc* sp., *Scytonema* sp	(Sharma et al., 2013)

Table 1-2. A summary of different organic acids produced by Phosphate solubilizing microorganisms (PSMs).

PSM	Organic acids	References
Bacillus sp.	Citric acid, malic acid, succinic acid, fumaric acid, tartaric acid, gluconic acid	(Selvi et al., 2017)
Pseudomonas sp.	Citric acid, succinic acid, fumaric acid, gluconic acid, 2-ketogluconic acids	(Kumar et al., 2018; Selvi et al., 2017)
Proteus sp.	Citric acid, succinic acid, fumaric acid, gluconic acid	(Selvi et al., 2017)
Aspergillus	Citric acid, gluconic acid, oxalic acid, succinic acid, malic acid, glycolic acid	(Sane & Mehta 2015)
Azospirillum sp.	Citric acid, succinic acid, fumaric acid, gluconic acid	(Selvi et al., 2017)
Penicillium sp.	Gluconic acid, glycolic acid, succinic acid, malic acid, oxalic acid, citric acid	(Sane & Mehta, 2015)
Erwinia herbicola	Gluconic acid, 2-ketogluconic acids	(Kumar et al., 2018)

REFERENCES

Abaidoo, R. C., Keyser, H. H., Singleton, P. W., Dashiell, K. E., & Sanginga, N. 2007. Population size, distribution, and symbiotic characteristics of indigenous *Bradyrhizobium* spp. that nodulate TGx soybean genotypes in Africa. *Appl. Soil Ecol.*, **35**, 57–67.

Abd-All, M., Issa, A. A., & Ohyam, T. 2014. Impact of Harsh Environmental Conditions on Nodule Formation and Dinitrogen Fixation of Legumes. *Adv. Biol. Ecol. Nitrogen Fixat.*, *January*.

Abd-Alla, M. H. 1994. Solubilization of rock phosphates by *Rhizobium* and *Bradyrhizobium*. *Folia Microbiol. Off. J. Inst. Microbiol. Acad. Sci. Czech Repub.*, **39**, 53–56.

Antoun, H., Beauchamp, C. J., Goussard, N., Chabot, R., & Lalande, R. 1998. Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: Effect on radishes (Raphanus sativus L.). *Plant Soil*, **204**, 57–67.

Babalola, O. O., & Glick, B. R. 2012. The use of microbial inoculants in African agriculture: Current practice and future prospects. *J. Food, Agric. Environ.*, **10**, 540–549.

Berman-Frank, I., Lundgren, P., & Falkowski, P. 2003. Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. *Res. Microbiol.*, **154**, 157–164.

Binns, A. N. 2002. T-DNA of *Agrobacterium tumefaciens*: 25 Years and counting. *Trends Plant Sci.*, **7**, 231–233.

Bore, E., Langsrud, S., Langsrud, Ø., Rode, T. M., & Holck, A. 2007. Acid-shock responses in *Staphylococcus aureus* investigated by global gene expression analysis. *Microbiology*, **153**, 2289–2303.

Brockwell, J., & Bottomley, P. J. 1995. Recent advances in inoculant technology and prospects for the future. *Soil Biol. Biochem.*, **27**, 683–697.

Brockwell, J., Roughley, R. J., & Herridge, D. F. 1987. Population dynamics of *Rhizobium japonicum* strains used to inoculate three successive crops of soybean. *Aust. J. Agric. Res.*, **38**, 61–74.

Brown, C. M., & Dilworth, M. J. 1975. Ammonia assimilation by *Rhizobium* cultures and bacteroids. *J. Gen. Microbiol.*, **86**, 39–48.

Cai, W. J., Hu, X., Huang, W. J., Murrell, M. C., Lehrter, J. C., Lohrenz, S. E., Chou, W. C., Zhai, W., Hollibaugh, J. T., Wang, Y., Zhao, P., Guo, X., Gundersen, K., Dai, M., & Gong, G. C. 2011. Acidification of subsurface coastal waters enhanced by eutrophication. *Nat. Geosci.*, **4**, 766–770.

Carpenter, S. R. 2005. Eutrophication of aquatic ecosystems: Bistability and soil phosphorus. *Proc. Natl. Acad. Sci. U. S. A.*, **102**, 10002–10005.

Chabot, R., Beauchamp, J. chantal, Kloepper, W. J., & Antoun, H. 1999. Effect of Phosphorus on Root Colonization and Growth Promotion of Maize By Bioluminescent Mutants of Phosphate-. *Soil Biol. Biochem.*, **30**, 1615–1618.

Chang, W. S., Lee, H.-I., & Hungria, M. 2015. Principles of plant-microbe interactions: Microbes for sustainable agriculture. In B. Lugtenberg (Ed.), *Principles of Plant-Microbe Interactions: Microbes for Sustainable Agriculture*, 1–448. Springer International.

Chen, L. S., Figueredo, A., Villani, H., Michajluk, J., & Hungria, M. 2002. Diversity and symbiotic effectiveness of rhizobia isolated from field-grown soybean nodules in Paraguay.

Biol. Fertil. Soils, **35**, 448–457.

Chung, H., Park, M., Madhaiyan, M., Seshadri, S., Song, J., Cho, H., & Sa, T. 2005. Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. *Soil Biol. Biochem.*, **37**, 1970–1974.

Cotter, P. D., & Hill, C. 2003. Surviving the Acid Test: Responses of Gram-Positive Bacteria to Low pH. *Microbiol. Mol. Biol. Rev.*, **67**, 429–453.

Couto, C., Silva, L. R., Valentão, P., Velázquez, E., Peix, A., & Andrade, P. B. 2011. Effects induced by the nodulation with *Bradyrhizobium japonicum* on *Glycine max* (soybean) metabolism and antioxidant potential. *Food Chem.*, **127**, 1487–1495.

D'Haeze, W., & Holsters, M. 2002. Nod factor structures, responses, and perception during initiation of nodule development. *Glycobiology*, **12**, 79–105.

Dabrowsk, K. J., & Sosulski, F. W. 1984. Composition of free and hydrolyzable phenolic acids in defatted flours of ten oilseeds. *J. Agric. Food Chem.*, **32**, 128–130.

Danso, S. K. A., Keya, S. O., & Alexander, M. 1975. Protozoa and the decline of *Rhizobium* populations added to soil. *Can. J. Microbiol.*, **21**, 884–895.

Drevon, J. J., & Hartwig, U. A. 1997. Phosphorus deficiency increases the argon-induced decline of nodule nitrogenase activity in soybean and alfalfa. *Planta*, **201**, 463–469.

Duine, J. a, Frank, J., & Zeeland, J. K. V. a N. 1979. Glucose Dehydrogenase. *FEBS Lett.*, **108**, 3–6.

Erisman, J. W., Galloway, J., Seitzinger, S., Bleeker, A., & Butterbach-Bahl, K. 2011. Reactive nitrogen in the environment and its effect on climate change. *Curr. Opin. Environ.*

Sustain., **3**, 281–290.

Farber, J. M., & Pagotto, F. 1992. The effect of acid shock on the heat resistance of *Listeria monocytogenes*. *Lett. Appl. Microbiol.*, **15**, 197–201.

Filippelli, G. M. 2008. The global phosphorus cycle: Past, present, and future. *Elements*, **4**, 89–95.

Foster, W. J., & Hall, K. H. 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. *J. Bacteriol.*, **172**, 771–778.

Franck, W. L., Qiu, J., Lee, H. I., Chang, W. S., & Stacey, G. 2015. DNA microarray-based identification of genes regulated by NtrC in *Bradyrhizobium japonicum*. *Appl. Environ. Microbiol.*, **81**, 5299–5308.

Gage, D. 2017. Infection and Invasion of Roots by Symbiotic, Nitrogen-Fixing Rhizobia during Nodulation of Temperate Legumes. *Microbiol. Mol. Biol. Rev.*, **68**, 280–300.

Galloway, J. N., Dentener, F. J., Capone, D. G., Boyer, E. W., Howarth, R. W., Seitzinger, S. P., Asner, G. P., Cleveland, C. C., Green, P. A., Holland, E. A., Karl, D. M., Michaels, A. F., Porter, J. H., Townsend, A. R., Vo¨ro¨smarty, C. J., Vo¨ro, V., & Vo¨ro¨smarty, V. 2004. Nitrogen cycles: past, present, and futur. In *Biogeochemistry*,70 (2).

Geurts, R., & Bisseling, T. 2002. Rhizobium nod factor perception and signalling. *Plant Cell*, **14**, 239–250.

Goldstein, A. H. 1995. Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by gram negative bacteria. *Biol. Agric.*

Hortic., **12**, 185–193.

Goodson, M., & Rowbury, R. J. 1989a. Habituation to normally lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH value. *Lett. Appl. Microbiol.*, **8**, 77–79.

Goodson, M., & Rowbury, R. J. 1989b. Resistance of acid-habituated *Escherichia coli* to organic acids and its medical and applied significance. *Lett. Appl. Microbiol.*, **8**, 211–214.

Goodson, M., & Rowbury, R. J. 1991. RecA-independent resistance to irradiation with u.v. light in acid-habituated *Escherichia coli*. *J. Appl. Bacteriol.*, **70**, 177–180.

Goulding, K. W. T. 2016. Soil acidification and the importance of liming agricultural soils with particular reference to the United Kingdom. *Soil Use Manag.*, **32**, 390–399.

Halder, A. K., & Chakrabartty, P. K. 1993. Solubilization of inorganic phosphate by *Rhizobium*. *Folia Microbiol. (Praha).*, **38**, 325–330.

Hernández, G., Valdés-Ló Pez, O., Ramírez, M., Goffard, N., Weiller, G., Aparicio-Fabre, R., Fuentes, S. I., Erban, A., Kopka, J., Udvardi, M. K., Vance, C. P., & Robert, S. 2009. Global Changes in the Transcript and Metabolic Profiles during Symbiotic Nitrogen Fixation in Phosphorus-Stressed Common Bean Plants. *Plant Physiol. Ò*, **151**, 1221–1238.

Herridge, D. F., Peoples, M. B., & Boddey, R. M. 2008. Global inputs of biological nitrogen fixation in agricultural systems. *Plant Soil*, **311**, 1–18.

Herrmann, L., & Lesueur, D. 2013. Challenges of formulation and quality of biofertilizers for successful inoculation. *Appl. Microbiol. Biotechnol.*, **97**, 8859–8873.

Howarth, R. W. 2008. Coastal nitrogen pollution: A review of sources and trends globally and regionally. *Harmful Algae*, **8**, 14–20.

Hungria, M., Loureiro, M. F., Mendes, I. C., Campo, R. J., & Graham, P. H. 2005. Inoculant Preparation, Production and Application. *Nitrogen Fixat. Agric. For. Ecol. Environ.*, 223–253.

Hungria, Mariangela, & Vargas, M. A. T. 2000. Environmental factors affecting N2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *F. Crop. Res.*, **65**, 151–164.

Huo, H., Wang, M., Bloyd, C., & Putsche, V. 2011. Life-cycle assessment of energy and greenhouse gas effects of soybeanderived biodiesel and renewable fuels. *Biodiesel Fuels Reexamined*, **43**, 125–198.

Isidra-Arellano, M. C., Reyero-Saavedra, M. D. R., Sánchez-Correa, M. D. S., Pingault, L., Sen, S., Joshi, T., Girard, L., Castro-Guerrero, N. A., Mendoza-Cozatl, D. G., Libault, M., & Valdés-López, O. 2018. Phosphate deficiency negatively affects early steps of the symbiosis between common bean and Rhizobia. *Genes (Basel).*, **9**.

Jansen, A. C., Goosen, N., Wenzel, T. J., & Putte, P. V. A. N. D. E. 1988. Cloning of the gene encoding quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus*: Evidence for the presence of a second enzyme. *J. Bacteriol.***170 (5)**, 2121–2125.

Joseph, S., & Jisha, M. S. 2009. Buffering Reduces Phosphate Solubilizing Ability of Selected Strains of Bacteria. *Public Health*, **5**, 135–137.

Kalayu, G. 2019. Phosphate solubilizing microorganisms: Promising approach as biofertilizers. *Int. J. Agron.*, **2019**.

Kaneko, T., Nakamura, Y., Sato, S., Asamizu, E., Kato, T., Sasamoto, S., Watanabe, A., Idesawa, K., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C.,

Kohara, M., Matsumoto, M., Matsuno, A., Mochizuki, Y., Nakayama, S., Nakazaki, N., Shipmo, S., Sugimoto, M., Takeuch, C., Yamada, M., Tabata, S. 2000. *Complete Genome Structure of the Nitrogen-fixing Symbiotic Bacterium Mesorhizobium loti*. *DNA Res.,* 7, 331–338.

Kaneko, T., Nakamura, Y., Sato, S., Minamisawa, K., Uchiumi, T., Sasamoto, S., Watanabe, A., Idesawa, K., Iriguchi, M., Kawashima, K., Kohara, M., Matsumoto, M., Shimpo, S., Tsuruoka, H., Wada, T., Yamada, M., & Tabata, S. 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. *DNA Res.*, **9**, 189–197.

Khairnar, N. P., Misra, H. S., & Apte, S. K. 2003. Pyrroloquinoline-quinone synthesized in *Escherichia coli* by pyrroloquinoline-quinone synthase of *Deinococcus radiodurans* plays a role beyond mineral phosphate solubilization. *Biochem. Biophys. Res. Commun.*, **312**, 303–308.

Khan, A. A., Jilani, G., Akhtar, s. M., Saqlan, M. S., & Rasheed, N. M. 2009. Phosphorus Solubilizing Bacteria: Occurrence, Mechanisms and their Role in Crop Production. *J. Agric. Biol. Sci.*, **1**, 48–58.

Khan, S. M., Zaidi, A., Ahemad, M., Oves, M., & Wani, A. P. 2010. Plant growth promotion by phosphate solubilizing fungi - Current perspective. *Arch. Agron. Soil Sci.*, **56**, 73–98.

Khosro, M., & Yousef, S. 2012. Bacterial Biofertilizers for Sustainable Crop Production : a Review. *J. Agric. Bol. Sci.*, **7**, 307–316.

Knobeloch, L., Salna, B., Hogan, A., Postle, J., & Anderson, H. 2000. Blue babies and nitrate-contaminated well water. *Environ. Health Perspect.*, **108**, 675–678.

Kosslak, R. M., Bookland, R., Barkei, J., Paaren, H. E., & Appelbaum, E. R. 1987. Induction of *Bradyrhizobium japonicum* common nod genes by isoflavones isolated from Glycine max. *Proc. Natl. Acad. Sci.*, **84**, 7428–7432.

Kousa, S., Labidi, N., Debez, A., & Abdelly, C. 2005. Effect of P on nodule formation and N fixation in bean. *Agron. Sustain. Dev.*, **25**, 389–393.

Kumar, A., Kumar, A., & Patel, H. 2018. Role of Microbes in Phosphorus Availability and Acquisition by Plants. *Int.J.Curr.Microbiol.App.Sci*, **7**, 1344–1347.

Lang, K., Lindemann, A., Hauser, F., & Göttfert, M. 2008. The genistein stimulon of *Bradyrhizobium japonicum*. *Mol. Genet. Genomics*, **279**, 203–211.

Lebrazi, S., & Benbrahim, K. F. 2014. Environmental stress conditions affecting the N2 fixing Rhizobium-legume symbiosis and adaptation mechanisms. *African J. Microbiol. Res.*, **8**, 4053–4061.

Lee, H. I., Lee, J. H., Park, K. H., Sangurdekar, D., & Chang, W. S. 2012. Effect of soybean coumestrol on *Bradyrhizobium japonicum* nodulation ability, biofilm formation, and transcriptional profile. *Appl. Environ. Microbiol.*, **78**, 2896–2903.

Li, J. H., Wang, E. T., Chen, W. F., & Chen, W. X. 2008. Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China. *Soil Biol. Biochem.*, **40**, 238–246.

Lin, J., Smith, M. P., Chapin, K. C., Baik, H. S., Bennett, G. N., & Foster, J. W. 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. *Appl. Environ. Microbiol.*, **62**, 3094–3100.

Mabrouk, Y., & Omrane, B. 2012. Enhancing the biological nitrogen fixation of leguminous crops grown under stressed environments. *African J. Biotechnol.*, **11**, 10809–10815.

Majumdar, D. 2003. The Blue Baby Syndrome: Nitrate poisoning in humans. *Blue Baby Syndr. Nitrate Poisoning Humans*, **8**, 20–30.

Mazur, W. M., Duke, J. A., Wähälä, K., Rasku, S., & Adlercreutz, H. 1998. Isoflavonoids and Lignans in Legumes: Nutritional and Health Aspects in Humans 11The method development and synthesis of the standards and deuterium-labelled compounds was supported by National Institutes of Health Grants No. 1 R01 CA56289-01 and No. 2 R0. *J. Nutr. Biochem.*, **9**, 193–200.

Mogk, A., Schlieker, C., Friedrich, K. L., Schönfeld, H. J., Vierling, E., & Bukau, B. 2003. Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK. *J. Biol. Chem.*, **278**, 31033–31042.

Montañez, A., Danso, S. K. A., & Hardarson, G. 1995. The effect of temperature on nodulation and nitrogen fixation by five *Bradyrhizobium japonicum* strains. *Appl. Soil Ecol.*, **2**, 165–174.

Mpepereki, S., Javaheri, F., Davis, P., & Giller, K. E. 2000. Soyabeans and sustainable agriculture. *F. Crop. Res.*, **65**, 137–149.

Mulvaney, R. L., Khan, S. A., & Ellsworth, T. R. 2009. Synthetic Nitrogen Fertilizers Deplete Soil Nitrogen: A Global Dilemma for Sustainable Cereal Production. *J. Environ. Qual.*, **38**, 2295–2314.

Naveed, M. 2016. The Life History of Pyrroloquinoline Quinone (PQQ): A Versatile Molecule

with Novel Impacts on Living Systems. *Int. J. Mol. Biol.*, **1**, 29–46.

Oke, V., & Long, S. R. 1999. Bacteroid formation in the Rhizobium-legume symbiosis. *Curr. Opin. Microbiol.*, **2**, 641–646.

Omoni, A. O., & Aluko, R. E. 2005. Soybean foods and their benefits: Potential mechanisms of action. *Nutr. Rev.*, **63**, 272–283.

Osa-Afiana, L. O., & Alexander, M. 1982. Differences Among Cowpea Rhizobia in Tolerance to High Temperature and Desiccation in Soil. *Appl. Environ. Microbiol.*, **43**, 435–439.

Peoples, M. B., Brockwell, J., Herridge, D. F., Rochester, I. J., Alves, B. J. R., Urquiaga, S., Boddey, R. M., Dakora, F. D., Bhattarai, S., Maskey, S. L., Sampet, C., Rerkasem, B., Khan, D. F., Hauggaard-Nielsen, H., & Jensen, E. S. 2009. The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis*, **48**, 1–17.

Pérez, E., Sulbarán, M., Ball, M. M., & Yarzábal, L. A. 2007. Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region. *Soil Biol. Biochem.*, **39**, 2905–2914.

Pierre, O., Engler, G., Hopkins, J., Brau, F., Boncompagni, E., & Hérouart, D. 2013. Peribacteroid space acidification: A marker of mature bacteroid functioning in *Medicago truncatula* nodules. *Plant, Cell Environ.*, **36**, 2059–2070.

Puehringer, S., Metlitzky, M., & Schwarzenbacher, R. 2008. The pyrroloquinoline quinone biosynthesis pathway revisited: A structural approach. *BMC Biochem.*, **9**.

Rabalais, N. N., Turner, R. E., Sen Gupta, B. K., Boesch, D. F., Chapman, P., & Murrell,

M. C. 2007. Hypoxia in the northern Gulf of Mexico: Does the science support the plan to reduce, mitigate, and control hypoxia? *Estuaries and Coasts*, **30**, 753–772.

Rodríguez, H., & Fraga, R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv.*, **17**, 319–339.

Ronner, E., Franke, A. C., Vanlauwe, B., Dianda, M., Edeh, E., Ukem, B., Bala, A., van Heerwaarden, J., & Giller, K. E. 2016. Understanding variability in soybean yield and response to P-fertilizer and rhizobium inoculants on farmers' fields in northern Nigeria. *F. Crop. Res.*, **186**, 133–145.

Sadowsky, M. J. 2006. Soil Stress Factors Influencing Symbiotic Nitrogen Fixation. *Nitrogen Fixat. Agric. For. Ecol. Environ.*, 89–112.

Sane, S. A., & Mehta, S. K. 2015. Isolation and Evaluation of Rock Phosphate Solubilizing Fungi as Potential Biofertilizer. *J. Fertil. Pestic.*, **06**.

Sanginga, N., Abaidoo, R., Dashiell, K., Carsky, R. J., & Okogun, A. 1996. Persistence and effectiveness of rhizobia nodulating promiscuous soybeans in moist savanna zones of Nigeria. *Appl. Soil Ecol.*, **3**, 215–224.

Sashidhar, B., & Podile, A. R. 2010. Mineral phosphate solubilization by rhizosphere bacteria and scope for manipulation of the direct oxidation pathway involving glucose dehydrogenase. *J. Appl. Microbiol.*, **109**, 1–12.

Scavia, D., & Bricker, S. B. 2006. Coastal eutrophication assessment in the United States. *Biogeochemistry*, **79**, 187–208.

Selvi, K., Paul, J., Vijaya, V., & Saraswathi, K. 2017. Analyzing the Efficacy of Phosphate

Solubilizing Microorganisms by Enrichment Culture Techniques. *Biochem. Mol. Biol. J.*, **03**, 1.

Sharma, B. S., Sayyed, Z. R., Trivedi, H. ., & Gobi, A. T. 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *J. Clin. Microbiol.*, **35**, 3305–3307.

Shridhar, B. S. 2012. Review : Nitrogen Fixing Microorganisms. *Int. J. Microbiol. Res.*, **3**, 46–52.

Silva, L. R., Pereira, M. J., Azevedo, J., Mulas, R., Velazquez, E., Gonzalez-Andres, F., Valentao, P., & Andrade, P. B. 2013. Inoculation with *Bradyrhizobium japonicum* enhances the organic and fatty acids content of soybean (*Glycine max* (L.) Merrill) seeds. *Food Chem.*, **141**, 3636–3648.

Srinivasan, R., Yandigeri, M. S., Kashyap, S., & Alagawadi, A. R. 2012. Effect of salt on survival and P-solubilization potential of phosphate solubilizing microorganisms from salt affected soils. *Saudi J. Biol. Sci.*, **19**, 427–434.

Stougaard, J. 2000. Regulators and regulation of legume root nodule development. *Plant Physiol.*, **124**, 531–540.

Subramanian, S., Stacey, G., & Yu, O. 2007. Distinct, crucial roles of flavonoids during legume nodulation. *Trends Plant Sci.*, **12**, 282–285.

Thuita, M., Pypers, P., Herrmann, L., Okalebo, R. J., Othieno, C., Muema, E., & Lesueur, D. 2012. Commercial rhizobial inoculants significantly enhance growth and nitrogen fixation of a promiscuous soybean variety in Kenyan soils. *Biol. Fertil. Soils*, **48**, 87–96.

Tilak, K. V. B. R., Ranganayaki, N., & Manoharachari, C. 2006. Synergistic effects of plant-growth promoting rhizobacteria and *Rhizobium* on nodulation and nitrogen fixation by pigeonpea (*Cajanus cajan*). *Eur. J. Soil Sci.*, **57**, 67–71.

Tzfira, T., & Citovsky, V. 2002. Partners-in-infection: Host proteins involved in the transformation of plant cells by Agrobacterium. *Trends Cell Biol.*, **12**, 121–129.

Vairinhos, F., Bhandari, B., & Nicholas, D. J. D. 1983. Planta and in bacteroids from root nodules of *Glycine max*. *Growth (Lakeland)*, 207–215.

Valverde, C., Ferrari, A., & Wall, L. G. 2002. Phosphorus and the regulation of nodulation in the actinorhizal symbiosis between *Discaria trinervis* (Rhamnaceae) and Frankia BCU110501. *New Phytol.*, **153**, 43–51.

Vassilev, N., Vassileva, M., & Nikolaeva, I. 2006. Simultaneous P-solubilizing and biocontrol activity of microorganisms: Potentials and future trends. *Appl. Microbiol. Biotechnol.*, **71**, 137–144.

Vitousek, P. M., Cassman, K., Cleveland, C., Crews, T., Field, C. B., Grimm, N. B., Howarth, R. W., Marino, R., Martinelli, L., Rastetter, E. B., & Sprent, J. I. 2002. Towards an ecological understanding of biological nitrogen fixation. *Biogeochemistry*, **57–58**, 1–45.

Walpola, charana B., & Yoon, M.-H. 2012. Prospectus of phosphate solubilizing microorganisms and phosphorus availability in agricultural soils: A review. *African J. Microbiol. Res.*, **6**, 6600–6605.

Whitehead, L. F., & Day, D. A. 1997. The peribacteroid membrane. *Physiol. Plant.*, **100**, 30–

44.

Williams, C. H. 1980. Soil acidification under clover pasture. *Aust. J. Exp. Agric.*, **20**, 561–567.

Xu, Y., Zhao, Z., Tong, W., Ding, Y., Liu, B., Shi, Y., Wang, J., Sun, S., Liu, M., Wang, Y., Qi, Q., Xian, M., & Zhao, G. 2020. An acid-tolerance response system protecting exponentially growing *Escherichia coli*. *Nat. Commun.*, **11**, 1469.

Yousefi, A. A., Khavazi, K., Moezi, A. A., Rejali, F., & Nadian, H. A. 2011. Phosphate Solubilizing Bacteria and Arbuscular Mycorrhizal Fungi Impacts on Inorganic Phosphorus Fractions and Wheat Growth. *World Appl. Sci. J.*, **15**, 1310–1318.

Zahran, H. H. 1999. Rhizobium-Legume Symbiosis and Nitrogen Fixation under Severe Conditions and in an Arid Climate. *Microbiol. Mol. Biol. Rev.*, **63**, 968–989.

Zhang, F., Dashti, N., Hynes, R. K., & Smith, D. L. 1996. Plant growth promoting rhizobacteria and soybean [*Glycine max* (L.) Merr.] nodulation and nitrogen fixation at suboptimal root zone temperatures. *Ann. Bot.*, **77**, 453–460.

Zhu, F., Qu, L., Hong, X., & Sun, X. 2011. Isolation and Characterization of a Phosphate-Solubilizing Halophilic Bacterium *Kushneria* sp. *YCWA18 from Daqiao Saltern on the Coast of Yellow Sea of China*. **2011**.

CHAPTER 2

PHYSIOLOGICAL RESPONSES OF THE SOYBEAN SYMBIONT *BRADYRHIZOBIUM JAPONICUM* TO HEAT STRESS

ABSTRACT

Tremendous growth of human population and degradation of arable lands, due to industrialization and urbanization, have led to food insecurity worldwide. Accelerating demand for food will keep rising; hence, scientists and agronomists are probing for promising alternatives to enhance crop production and ensure food security in a sustainable manner. Biofertilizers mark a milestone in this journey for improved crop yields with a healthy soil environment. Commercially available inoculants of rhizobia and bradyrhizobia are examples of commonly used biofertilizers to improve soil nitrogen (N) fertility. The soybean symbiont *Bradyrhizobium japonicum* is capable of N fixation while residing in the root nodules to enhance the plant growth as a personalized source of N. Hence, the soybean-*Bradyrhizobium* symbiosis is significant as the most economical way of N fertilization to mitigate adverse effects of synthetic nitrogen fertilizers. However, failure to tolerate and survive the abiotic stress conditions in the field such as extreme temperatures (e.g., heat stress) is the major constraint of biofertilizer industry. Therefore, it is important to understand how these bacteria respond to stress factors so that we could explore the potential avenues for formulation of robust rhizobial inoculants. In this study, genetics, and physiology of *B. japonicum* was investigated under heat stress using genome wide transcriptional profiling. Growth curve analysis conducted under different heat stress conditions showed that exposure to 42°C for 30 min would be the stress condition under which the cells can be still grown even at slower rate. Whole genome transcriptional profiling under this condition using microarray analysis showed that 621 and 515

genes were up and down regulated, respectively. Mutagenesis studies performed on highly up-regulated genes under heat stress to deduce their ecological functions and symbiotic roles showed that small heat shock protein coding gene *blr7740* and RNA polymerase sigma factor coding gene *blr2203* are important neither for normal growth nor survival under heat shock. Loss of these gene activities might be compensated by other genes.

INTRODUCTION

Bacterial heat shock response is mainly controlled by the alternative sigma factor RpoH (σ^{32}) (Guisbert et al., 2004; Martínez-Salazar et al., 2009). Most bacterial genomes that have been sequenced up to date have reported the genes encoding RpoH and it recognizes a different promoter region than the housekeeping sigma factor RpoD (σ^{70}). RpoH of *Escherichia coli* control the expression of about 91 genes encoding chaperones (GroEL, GroES, DnaK, DnaJ and GrpE) and proteases (FtsH and Lon). RpoH is also reported to be involved in oxidative response, symbiosis, and pathogenicity of some other bacteria in addition to heat stress response.

Generally, bacterial genomes possess only a single *ropH* gene. However, some α-proteobacteria have two or three *rpoH* homologues. Two *rpoH* genes have been identified in *Brucella melitensis, Rhodobacter sphaeroides*, and in the nitrogen-fixing symbionts *Mesorhizobium loti, Sinorhizobium meliloti* and *Rhizobium etli* (Galibert et al., 2001; González et al., 2006; Kaneko et al., 2000; Martínez-Salazar et al., 2009) and *B. japonicum* genome contains three *rpoH* like genes (Kaneko et al., 2002). These are proved to be functionally similar to *E. coli* σ^{32} (Delory et al., 2006; Green & Donohue, 2006; Narberhaus et al., 1997). The importance of having multiple *rpoH* genes is not yet understood. Even though, *rpoH2* gene was confirmed to be essential for growth while *rpoH1* and *rpoH3* genes did not significantly affect the aerobic growth and symbiosis during mutagenesis studies. However, the gene expression and the complementation

studies done with RpoH mutated *E. coli* proposed that *rpoH1* and *rpoH3* genes are involved in the heat shock response (Narberhaus et al., 1997). In *R. etli* both *rpoH1* and *rpoH2* were able to complement temperature sensitive *E. coli rpoH* mutant. However, further studies concluded that, RpoH1 is the main heat shock sigma factor in *R. etli* and together with RpoH2 more complete protective response is achieved. Additionally, both *rpoH1* and *rpoH2* elicited have a positive effect on nitrogenase activity and productive life span of root nodules (Martínez-Salazar et al., 2009). Both eukaryotes and prokaryotes respond to environmental stress conditions such as elevated temperatures by an increase production of a special group of proteins called heat shock proteins (Hsps). When the temperature is shifted, transiently increase the rate of synthesis of Hsps than the other proteins. Generally, few of these Hsps are required by the cells for growth at all temperatures. However, the demand will be increased at elevated temperatures. Response to higher temperatures through Hsps is not a unique process for bacteria but found in eukaryotes as well. Heat shock stress caused by a sudden temperature increase affect the cellular protein homeostasis. Major functions of the Hsps are to assist the folding of newly synthesized proteins, assembly, transport, and degradation of proteins during normal growth; especially under stress conditions (Minder et al., 2000). Under heat stress, these Hsps prevent aggregation of proteins and recover partially or completely unfolded proteins (Roncarati & Scarlato, 2017). Hsps can be chaperones, proteases, and small heat shock proteins. There are six classes of proteins that have chaperone activity: Hsp100, Hsp 90, Hsp 70, Hsp 60, Hsp 40, and the small heat shock proteins (sHsp). Hsp 100 proteins involve in ATP-dependent disaggregation and unfolding for degradation, promotion of proteolysis of specific cellular substrates and regulation of transcription. Hsp 90 prevent incorrect interactions of proteins; have a major role in cellular processes such as hormone signaling and cell cycle. Hsp 70 family consists of DnaK, Hsp 60 family typified by GroEL and Hsp40 family

contains DnaJ are well characterized for their structure and functions as chaperones. Hsps, GroEL, and DnaK are designated as "molecular chaperones" for their functions that are folding of newly synthesized proteins and refolding of denatured proteins. Both GroEL and DnaK chaperones require the assistance of co-chaperones GroES and DnaJ/GrpE respectively for their proper functioning in proper folding of the substrate polypeptides. Chaperones are responsible for cell protection against heat stress and the growth under normal conditions as well (Babst et al., 1996). Small heat shock proteins (sHsps or α-Hsps) are another family of molecular chaperones. Although, sHsps have a low molecular weight between 12 and 43 kDa they usually aggregate to form larger, high molecular weight oligomers with several subunits. This is a highly diverse family of heat shock proteins which is sharing a conserved α-crystallin homology domain. sHsps also exhibit chaperone activity by forming stable complexes with folding intermediates of their protein substrates. Chaperone activity of sHsps is ATP-independent and hence they are lacking refolding activity. Upon binding with sHsps, denatured proteins are maintained at a stable and refoldable state until subsequent refolding by ATP-dependent chaperones such as DnaK and ClpB (Mogk et al., 2003; Veinger et al., 1998). Most bacteria have few members of Hsp per class while rhizobia tend to have a set of multigene heat shock protein families like GroESL and sHsps (Fischer et al., 1993; Michiels et al., 1994). B. japonicum possess at least five *groESL* and seven shsp genes. B. japonicum proved to be a model organism to study about the function of heat shock genes due its uniqueness in sHsps induction pattern. Bacterial sHsps are divided into two classes: class A consisting of proteins that are like *E. coli IbpA* and *IbpB* and class B contains proteins 8 like other sHsps from prokaryotes and eukaryotes. *B. japonicum* contains both classes and recent complete genome sequencing studies have shown that 11 shsps genes: seven coding for class A and four coding for class B proteins (Kaneko et al., 2002). It was found the proteins from both classes form

large complexes to protect citrate synthase from heat shock in the laboratory experiments (Studer & Narberhaus, 2000). Much of the explanations about bacterial heat shock response regulation have been based on the *E. coli*. In these species alternative sigma factors are involved in directing the RNA polymerase to heat shock gene promoters. Following a temperature upshift (30°C-42°C), there will be a significant increase in the concentration of sigma factor σ32 which is also called RpoH responsible for the synthesis of at least 30 Hsps acting in cytoplasm. σ32 recognizes the gene promoters in a major heat shock regulon the σ32 regulon. There are several factors that lead to accumulation of σ32 at higher temperatures. During steady growth rate at any temperature σ32 is an unstable protein with a half-life of one minute. Following temperature shifting from 30°C to 42°C, this protein will be stabilized for few minutes while increasing its amount in the cell. At non- stress temperatures (30°C), there is a free cytoplasmic pool of DnaK and DnaJ available. Therefore, DnaK and DnaJ could bind with the σ32 factor which will result in proteolysis of σ32 factor by proteases such as FtsH, Hs1VU, ClpAP and Lon. At high temperatures (42°C), DnaK and DnaJ in the free cytoplasmic pool preferentially bind to denatured proteins and σ32 factor binds to RNA polymerase. Binding of σ32 to RNA polymerase protects the sigma factor from proteolysis and forms the holoenzyme that transcribes the Hsp σ 32 regulon. The activity and the accumulation of the σ32 factor is increased by the number of denatured proteins in the cell. Additionally, there is an increase of translation of the mRNA for σ 32 during higher temperatures. After, downshift of temperature the activity of σ32 decreases by lowering the rate of Hsps synthesis (Narberhaus, 1999). The σE or σ24 is another regulon activated by heat stress caused by very high temperatures like 45°C to 50°C. It provides the protection against the damage happens to extracytoplasmic proteins. Stress conditions created by high temperatures like 45°C to 50°C or ethanol result in the denaturation of outer membrane or periplasmic proteins. Denatured proteins

will be the stimulant to activate the σE factor in cytoplasm. Consequently, σE regulon consists of 11 genes is transcribed. The proteins synthesized by this regulon includes a periplasmic peptidylprolyl isomerase (FkpA) and a periplasmic protease (DegP) which involves in folding, refolding or degradation of misfolded envelope proteins (Missiakas & Raina, 1997).

Three different regulatory systems including both positive and negative regulatory mechanisms that control transcription of heat shock genes have been identified in *B. japonicum* (Narberhaus, 1999) (Fig. 2-1). One class of heat shock genes is transcribed from σ 32 dependent promoters (Narberhaus et al., 1997). The second class of heat shock genes is negatively controlled by a highly conserved DNA element called ROSE (Repression of heat shock gene express) (Narberhaus et al., 1998). The third group of genes is negatively controlled by the presence of a DNA element called CIRCE (controlling inverted repeat of chaperone expression) in the promoter region of groESL4 and groESL5 chaperonin operons (Babst et al., 1996; Narberhaus et al., 1997).

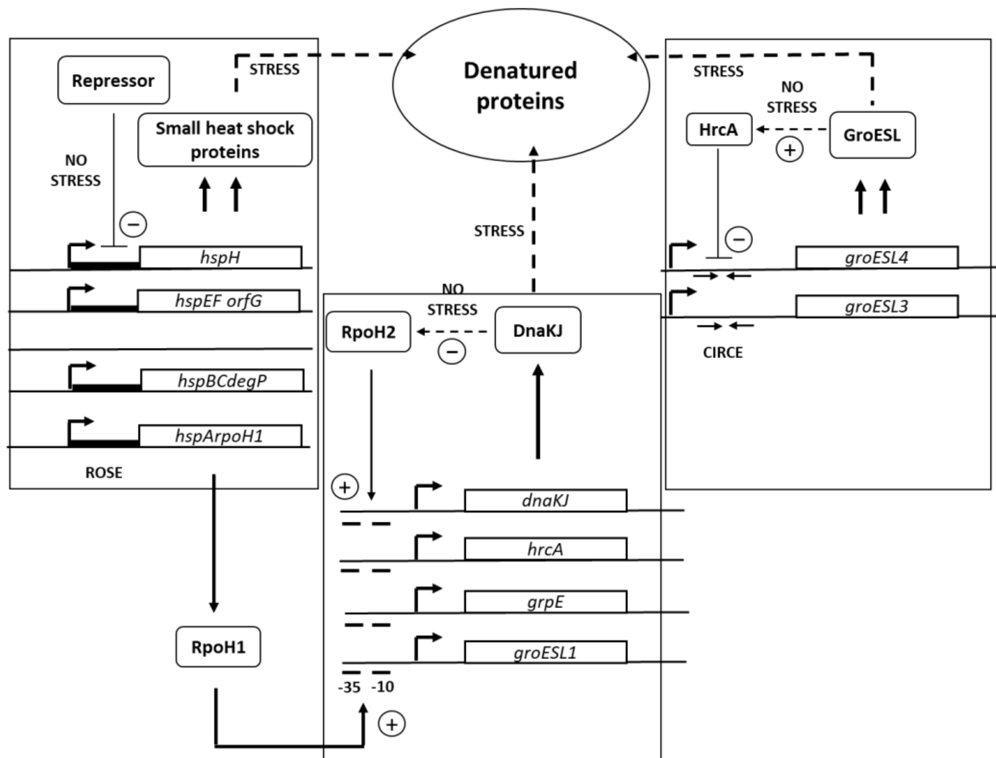

Figure 2- 1. An illustration showing the three regulatory mechanisms of heat shock gene response in *B. japonicum*.

The purpose of this study was to earn more detailed understanding of the complex heat shock regulatory system of *B. japonicum* using whole genome transcriptomic profiling. The aim of this study is to deduce ecological and symbiotic functions of significantly regulated genes in response to heat shock and to evaluate possible means of improving symbiotic performances of the bacterium in the field.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 2-1. The wild type strain of *Bradyrhizobium japonicum* USDA110 was cultured in arabinose-gluconate (AG) medium at pH 6.8 which contains 125 mg of Na_2HPO_4, 250 mg of Na_2SO_4, 320 mg of NH_4Cl, 180 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2$, 4 mg of $FeCl_3$, 1.3 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1g of 2-(N-morpholino) ethanesulfonic acid (MES), 1 g of yeast extract, 1 g of L-arabinose, and 1 g of D-gluconic acid sodium sulfate per L of distilled water (Sadowsky et al., 1987). *Bradyrhizobium japonicum* USDA110 was incubated aerobically at 30°C with shaking at 200 rpm when grown in liquid media. Antibiotics used for strain selection were chloramphenicol (50 $\mu g ml^{-1}$ for wild type strain of *Bradyrhizobium japonicum* USDA110 and mutants) and kanamycin (150 $\mu g ml^{-1}$ for mutant strains). *Escherichia coli* strains and plasmids were grown aerobically in Luria-Bertani (LB) medium at pH 7.0 which contains 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of distilled water (Bertani, 1951). All *Escherichia coli* cultures were grown at 37°C (except DH5α pKD78 incubated at 30°C), with shaking at 200 rpm overnight in liquid media. Appropriate antibiotics were added into media when needed as follows: gentamicin (15 $\mu g ml^{-1}$ for pJQ200SK),

chloramphenicol (15 μgml^{-1} for pKD78) and kanamycin (50 μgml^{-1} for pRK2013 and pKD4). When preparing agar plates of each medium, 15 g of agar was added per L of distilled water.

Heat stress assay. *B. japonicum* USDA110 cultures were grown in AG media at 30°C aerobically with vigorous shaking at 200 rpm until mid-log phase. Subcultures of 100 ml each with chloramphenicol were grown at 30°C aerobically with shaking at 200 rpm until reached exponential phase with OD_{600nm} of 0.8-1.0. Culture was mixed well and divided into 50 ml each in 250 ml flasks for heat shock treatment. To provide the heat shock condition to bacteria one flask with 50 ml culture was incubated at different temperatures (15°C, 42°C & 48°C) for different 30 mis. while the other 50 ml was maintained at 30°C as the control group. The cultures were incubated for 2 h at 30°C with shaking at 200 rpm. Each treatment was replicated three times. OD_{600nm} and CFU/ ml were measured before (at 0 min) and after (10, 30, 60 and 90 min) the heat shock treatment.

RNA isolation. Total RNA was isolated from *B. japonicum* USDA110 cultures grown in AG medium under heat stress at 42°C for 30 min. 100 ml of *B. japonicum* USDA110 culture was grown at 30°C with shaking at 200 rpm until it reaches OD 600_{nm} 0.8. Then 50 ml of the culture was incubated at 42°C for 30 min. under heat stress and the other 50 ml of the culture was maintained at 30°C as the control group. The experiment was conducted in three biological replicates. RNA extraction was performed using hot phenol method as previously described (Jeon et al, 2011)**.** All cultures were condensed by centrifugation for 20 min at 4°C and 4,000 rpm in a fixed angle rotor. The cell pellet was collected by decanting the supernatant and stored at -80°C until use. The RNeasy mini kit (Qiagen) and RNase-free DNase (Qiagen) was used to purify the isolated RNA according to manufacturer's protocol. RNA concentration was measured on NanoDrop (Thermo Scientific) and RNA quality was checked using gel electrophoresis.

Gene expression analysis by microarray hybridization. Whole-Genome transcriptional profiles of *B. japonicum* USDA110 in response to heat stress were created from the hybridization of cDNA samples labeled with Cy3 and Cy5 monoreactive dyes (GE Healthcare) to microarray chips containing 70-mer oligonucleotides that were complementary to each of the 8,453 annotated open reading frames (ORFs) of *B. japonicum* (Chang et al., 2007). Thirty micrograms of total RNA was used for cDNA synthesis, and 5 µg of cDNA from both control and experimental conditions was used for labeling and hybridization. The detailed protocols for cDNA synthesis, cDNA labeling, hybridization, and washing have been described previously (Chang et al., 2007). A total of three independent biological replicates were prepared for each condition, which included a dye-swap for each replicate, resulting in a total of 6 slides for each experimental condition.

Statistical analysis of microarray data. The slides were scanned with the Axon GenePix 4200 scanner, and GenePix Pro 6.0 software was used to measure intensity values at each spot. The signal intensities were normalized for slide and spot abnormalities using the locally weighted scatterplot smoothing (LOWESS) algorithm and subsequently analyzed by mixed-effect microarray analysis of variance (MAANOVA) (Jeon et al., 2011). Values obtained from this round of analysis were input into a significance analysis of microarray (SAM) statistical package (Tusher et al., 2001) to create a list of differentially expressed genes with a fold induction threshold of 1.5 or 2.0 and a false-discovery rate (FDR) of 5% or less ($q <= 0.05$ [q is the adjusted P value by FDR-based multiple testing correction])

Construction of mutant strains. Two mutants were constructed using site-specific mutagenesis to delete the selected genes (*blr7740* and *blr2203*) that were upregulated in response to heat shock, from the genome of *B. japonicum* USDA110. A 1.46 kb fragment containing the *B. japonicum* USDA110, small heat shock protein coding gene, *blr7740*, was amplified by PCR using

forward primer *blr7740*_FW (5' ATTAGGATCCTATTTCTCGATCTGCTCGCT 3') and reverse primer *blr7740*_RV (5' ATATAGGGCCCTTCCGAGAGCTTGGTGTAG 3') which have restriction enzyme recognition linkers for BamHI (*blr7740*_FW) and ApaI (*blr7740*_RV) respectively at 5' ends. The *B. japonicum* USDA110, RNA polymerase sigma factor coding gene under heat shock, *blr2203*, was amplified as a 1.81kb long DNA fragment using forward primer *blr2203*_FW (5' ATATGGATCCGTCACGTGATACCCTCATTC 3') and reverse primer *blr2203*_RV (5' ATATAGGGCCCGAAGATCTGAAGCGACAGC 3') which have restriction enzyme recognition linkers for BamHI (*blr2203*_FW) and ApaI (*blr2203*_RV) respectively at 5' ends. PCR amplification was performed on C1000 Thermo Cycler (Biorad, Hercules, CA) followed by gel electrophoresis, using a PowerPacTM Basic (Biorad, Hercules, CA), to confirm the proper amplification of the target regions. Then, the target amplicons were inserted into the pJQ200SK (gentamycin resistant) suicide vector plasmid (Quandt & Hynes, 1993) through double digestion by the restriction enzymes; BamHI and ApaI followed by ligation. The recombinant pJQ200SK vector plasmid carrying the gene of interest was then introduced into DH5α competent cells via heat shock transformation. The vector plasmids with the clone were transformed into electrocompetent DH5α pKD78 (chloramphenicol resistance) cells carrying the Lambda Red Recombinase expression plasmid pKD78, via electroporation with Micro PulserTM (Biorad, Hercules, CA), at 1.8 kV. Kanamycin resistance cassette (1.4kb) was amplified from pKD4 (kanamycin resistance) plasmid by PCR using the forward primer (PS1): 5' GTGTAGGCTGGAGCTGCTTC 3' and reverse primer (PS2): 5' CATATGAATATCCTCCTTAG 3' (Datsenko and Wanner 2000). Then the kanamycin cassette was re-amplified with two 60-bp primers, which have 40-bp linkers homologous to both bordering regions of the deletion target at 5' ends of PS1 and PS2 using Phusion Taq polymerase enzyme.

For *blr7740* gene FW_60 bp primer: 5'CCCGGCCCGATTGGCGGGCGGGTTACCAAACAGTCGCTCAGTGTAGGCTGGAGCTGCTTC 3' and RV_60 bp primer: 5'CACCGGGGCGTTTTCGCAACCTGGAAGTGGCGTGCGTGTTCATATGAATATCCTCCTTAG 3' were used. For *blr2203* gene FW_60 bp primer: 5' CTGCGCGGAACGCGCCTGAGCCTACGCGTTGGGGGGCTGAGTGTAGGCTGGAGCTGCTTC 3' and RV_60 bp primer: 5' CATCACTTGCAGTCCAGAACCACCCTCACAGCATAAACCCCATATGAATATCCTCCTTAG 3' were used. The PCR product from the previous step was then electroporated using Micro Pulser™, at 2.2 kV into electrocompetent DH5α pKD78 cells containing targeted genomic clone, to induce λ Red genes mediated homologous recombination. Kanamycin resistant recombinants were selected and deletion constructs were confirmed by PCR using the forward or reverse primers from the initial PCR cloning in combination with one of the two primers located inside the kanamycin resistance gene, PSk1 (5' CAGTCATAGCCGAAT AGCCT 3') and PSkt′ (5' GGATTCATCGACTGTGGCCG 3') (Datsenko & Wanner, 2000). The resulting deletion constructs for each gene were transferred from DH5α pKD78 to the wild type *B. japonicum* USDA110 by tri-parental mating with the helper plasmid pRK2073. Transconjugants generated from double-crossover homologous recombination were selected with the help of *sacB* marker and resistance to kanamycin and chloramphenicol. The mutant strains were confirmed by PCR using the same primer combination used for deletion constructs confirmation.

Comparison of growth rate. *B. japonicum* USDA 110 (wild type), *blr7740* mutant and *blr2203* mutant strains were grown in 10 ml of AG medium (pH 6.8) at 30°C with shaking at 200 rpm for 2 days with proper antibiotics until reach mid-log phase and then subcultured in 50 ml of

AG media with proper antibiotics. Three replicates for each strain were incubated at 30°C with shaking at 200 rpm for 5 days until the cultures reach the stationary phase. OD600 measurements and CFU counting was performed in every 12 h interval during the experimental period. This experiment was repeated three times.

Survivability test under heat shock. The wild type, *blr7740* mutant and *blr2203* mutant strains were grown in 10 ml of AG medium (pH 6.8) at 30°C with shaking at 200 rpm with proper antibiotics until reach mid-log phase and then subcultured in 50 ml of AG media with proper antibiotics. Three replicates of each strain were incubated at 30°C with shaking at 200 rpm until the cultures reach OD600 0.8. Cultures at 0.8 OD600 were divided into two flasks and one flask of each strain was incubated at 42°C for 10, 20, 30, 60, 90, and 120 min. The other flask of each strain was incubated at 30°C and OD600 measurements and CFU counts of each strain at each time point were obtained.

RESULTS

Heat stress induced cell death and survivability. *B. japonicum* USDA110 that grown until mid-log phase at 30°C was exposed to higher temperatures instantaneously to study how it behaves in response to heat shock conditions. In this study 42°C and 48°C were selected as the experimental temperatures while 30°C served as the control. Growth of the bacterium was monitored, and number of viable cells were recorded in different time intervals after exposing to the selected high temperatures. Viable cell counts at selected time points confirmed that 48°C is too stressful for the bacterium showing rapid decline of number of cells after 30 min of exposure (Fig.2-3). Survivability% calculated at each time point were also consistent with that at 48°C cells are dying more rapidly than at 42°C (Fig.2-4). By considering the viable cell counts and

survivability % at each time point, 30 min of exposure to 42°C was finalized as the heat stress condition for *B. japonicum* USDA110 for this study.

Whole genome transcriptional analysis of *B. japonicum* USDA110 cells exposed to heat stress. Microarray analysis was done to study the gene expression of *B. japonicum* USDA110 in response to heat stress. To identify the genes that were specifically regulated in response to heat stress, gene expression of the cells exposed to 42°C for 30 min was compared to that of the cells maintained at 30°C for 30 min after reaching mid-log phase. Microarray analyses indicated that of the 8, 480 *B. japonicum* USDA110 ORFs analyzed 1136 were differentially expressed more than 2.0 cut-off ($p < 0.05$). Among them, 621 genes were uniquely up-regulated while 515 genes were significantly down-regulated in total. Genes that were significantly up-regulated and down-regulated in response to heat stress, can be categorized into 16 functional groups (Fig. 2-2). The highest number of stimulated and suppressed genes encode hypothetical proteins; hence, their specific physiological function could not be implied. However, a variety of genes encoding proteins involved in cellular processes, regulatory functions, translation, and transport were significantly regulated in response to heat stress. Among those groups, 49 genes involved in regulatory functions, 23 translation related genes and 44 transport and binding related genes were up-regulated while more cellular processes associated (37 genes) were down-regulated. There was no difference between the number of genes that were up and down regulated in relation to amino acid biosynthesis or energy metabolism. The same number of genes that are responsible for purines, pyrimidines, nucleosides, and nucleotides synthesis (7 genes) were up and down regulated. Transcription related genes were very lowly expressed comparatively to other functional groups where only 8 gene was up regulated while 5 genes were suppressed. Further, there was not much difference between the up and down regulated number of genes related to fatty acid,

phospholipid, and sterol metabolism where 13 genes were up-regulated while 10 genes were down-regulated. Surprisingly, no genes were regulated in relation to symbiosis under heat stress (Table 1.). There are several types of heat shock proteins among the highly up regulated top 20 genes such as small heat shock proteins, heat shock proteins, proteases, and chaperones. Small heat shock protein coding genes *blr7740* and *blr5221* were up regulated in 18.0 and 14.8 folds, respectively. Heat shock protein *blr5227* was expressed in 13.8 folds while *blr2450* protease heat shock protein was stimulated in 10.5 folds. Chaperone protein *blr0680* was regulated 11.9 times higher than the control. *blr7731*, inositol monophosphatase family protein coding gene is the gene with the second highest fold induction value following the hypothetical protein *bll5218* which has the highest fold induction of 35.6. Further, genes coding for other important ATP dependent proteases which involve in intracellular protein degradation such as ATP-dependent protease LA *blr6174* and ATP-dependent protease ATP-binding subunit *blr1404* were induced in 11.2 and 11.0 folds, respectively. Additionally, *blr2203* which is a putative RNA polymerase sigma factor coding gene was expressed in 10.2 folds and *bll3190* ABC transporter ATP-binding protein was induced in 10.3 folds. In addition to highly up regulated genes, there are many other genes that were induced to protect cells from heat stress such as amino acid and co-factor biosynthesis related genes.
When considering the down-regulated genes several genes related to flagella production such as *bll6866* and *bll6865* have been significantly down-regulated in 18.6 and 10.9 folds, respectively. Apart from that, several other genes that might be involved in energy production, cell division and nitrogen fixation have been suppressed.

Two highly up-regulated genes, small heat shock protein coding gene, *blr7740* (18.0 folds) and putative RNA polymerase sigma factor coding gene, *blr2203* (10.2 folds) were selected for mutagenesis studies. Growth curve comparisons showed that the two mutants are also growing at

the same rate as the wild type and that infers the selected two genes are not vital for the growth of *B. japonicum* USDA110 under normal conditions (Fig. 2-5, Fig. 2-6, Fig. 2-7 & Fig. 2-8). Survivability studies done with the wild type and the two mutants under heat stress indicated that there is no significance difference of the survivability of either mutant or the wild type (Fig. 2-9).

DISCUSSION:

This study was conducted to investigate the heat shock responses of *B. japonicum* USDA110, the N fixing microsymbiont of soybean plant. Utilization of symbiotic rhizobia to improve soil fertility is a well-established agricultural practice since decades. Increased demand for N and P biofertilizers in sustainable agriculture has promoted studies done on rhizobia diversity, performance, cellular responses, and tolerance to biotic and abiotic stress conditions such as temperature, pH, desiccation, and salinity. Fluctuations in temperature, specifically high temperatures greatly influence early stages of nodulation as well as the symbiotic performances inside the matured nodules. Additionally, temperature is a critical factor that governs the persistence of free-living rhizobia in soil. Bacterial heat stress response is probably the most examined stress response. In this study, a series of growth curve experiments was conducted to observe the behavior of *B. japonicum* USDA110 in response to different heat stress conditions, to find the stressful condition for the bacterium that should be used in the gene expression study. Results of the growth curve analysis showed that the bacterium is unable to survive upon sudden exposures to higher temperatures such as 48°C. Number of cells survived began to decline rapidly after 10 min of exposure to 48°C. Cells were able to survive in a decreased rate after exposing to 42°C. According to the survivability data under different temperatures at different time intervals indicated that the cells are completely dead after 30 min of exposing to 48°C leaving that 30 min at 42°C as the stressful condition for *B. japonicum* USDA110 under which it can thrive at a reduced

rate. That experimental condition was chosen to be used in gene expression analysis to explore what genes and mechanisms made it to subsist the stress condition.

Both extremes of temperature should be considered when comparing the efficiency and effectiveness of rhizobia in SNF. However, heat stress is the most prevalent abiotic stress associated with agricultural soil. The optimum range of temperature for most rhizobia is 25–31°C (Somasegaran et al., 1994) and the upper temperature limit is set between 32 and 47°C (Karanja & Wood, 1988; Munévar & Wollum, 1981). However, the critical range of temperature is species and strain dependent. For example, rhizobia nodulating common bean are able to grow at temperatures up to 44 °C (Diouf et al., 2000) and the maximum growth temperature for chickpea rhizobia is 40 °C for both *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* (Nour et al., 1994). Also, the sensitivity or tolerance of rhizobia to heat stress can be ameliorated through soil physical property adjustments. For example, *Bradyrhizobium* sp. (lupins) was less susceptible than *R. leguminosarum bv. trifolii* to high soil temperatures, and by addition of montmorillonite and illite this problem can be alleviated in sandy soils (Bushby & Marshall, 1977). One study conducted with three indigenous *B. japonicum* isolates in India concluded that they grow well at 25°C. However, the symbiosis itself is sensitive to low temperatures as well. (Lynch & Smith, 1994; Zhang et al., 1996).

The Heat-shock response of bacteria is associated with the induction of number of proteins called heat-shock proteins, or HSP in response to sudden variations of temperature. The HSPs consists of chaperones and proteases that are essential prevent and overcome protein denaturation happens due to temperature variations. In *Escherichia coli* the heat-shock response is controlled by a specific sigma factor-32 (σ32), this factor coded by the *rpoH* gene and binds to heat-shock promoters located upstream of heat shock genes (Straus et al., 1987). Many molecular chaperones

have developed to control protein folding and protection of the cell from elevated temperatures (Street et al., 2014). HSP include chaperones and proteases that are necessary for maintaining the cell, in abnormal condition such protein denaturation as well as under normal growth conditions.

Microarray hybridization studies showed that many genes related to heat shock protein production were stimulated in response to 30 min of exposure to 42℃ such as small heat shock protein coding genes *blr7740*, *blr5221*, *blr5222*, *blr5234*, *blr5230*, and *blr5233*. Also, heat shock proteins, proteases and chaperone proteins were also induced. The small heat-shock proteins constitute a highly diverse group of proteins whose expression is induced under stressful conditions. Their main responsibility is to bind and protect un-folded proteins, by holding them in a conformation to prevent degradation, until they are refolded by ATP-driven chaperones (Matuszewska et al., 2005).

Some of the heat-shock proteins are also required during normal growth condition and they are abundant under all metabolic conditions. GroEL and DnaK, are the examples for two major chaperone families Hsp60 and Hsp70, in bacteria, respectively. These proteins play a key role in protein folding even during non-stressed regular growth conditions. However, their production and action become more prominent during heat stress. They bind with hydrophobic surfaces of unfolded proteins and support them regain the proper folding, with the aid of their co-chaperones (GroES and DnaJ-GrpE) and ATP hydrolysis. These chaperones have different and complicated assembly of their monomers in a unique way to assist their functions. For example, GroEL monomers assemble as cylindrical complex to form two heptameric rings that enclose the entire substrate protein inside a large cavity, preventing interactions with other proteins during folding and the DnaK chaperone is mostly monomeric and bind with short surface-exposed hydrophobic amino-acid sequences (Mogk et al., 1999; Xu et al., 1997).

Another group of heat-shock proteins expressed and upregulated during stress in bacteria is proteases. These proteins are responsible for the removal of damaged polypeptides from stressed cells. Some proteases are multicomponent systems. They have a catalytic subunit (e.g. ClpP and HslV) associates to substrate recognition subunits (ClpA or ClpX for ClpP and HslU for HslV), which are co-chaperones able to remodel substrate polypeptides upon ATP hydrolysis and deliver them to proteolytic degradation (Missiakas et al., 1996; Wawrzynow et al., 1996). While these proteases assemble into complex ring-shaped structures, other members of this group of heat-shock proteins combine on a single polypeptide both chaperone and protease activities (e.g., Lon, FtsH and the periplasmic serine protease DegP). Additionally, some heat shock proteins have been identified as virulence factors that involve in pathogenesis of some pathogens (Bohne et al., 1994; Roncarati & Scarlato, 2017).

Growth studies done with the wild type and the two mutants showed that though the small heat shock protein coding gene *blr7740* and the RNA polymerase sigma factor coding gene *blr2203* were induced in higher folds under heat stress, they are not vital either for growth at normal conditions or under heat stress conditions. Being one of the several small heat shock proteins that were highly up regulated, loss of *blr7740* gene might have been compensated by the function of the other small heat shock proteins. Even though *blr2203* was coding for a RNA polymerase sigma factor that might be useful under heat stress, loss of that gene was also not deleterious for the survival of the bacterium in either conditions.

Bacterial heat shock response is controlled by the alternative sigma factor RpoH (σ32) at the transcriptional level (Arsène et al., 2000; Martínez-Salazar et al., 2009). Majority of bacterial genomes have found to contain genes encoding RpoH. The RpoH sigma factors recognize a different promoter region than the promoter that is recognized by the housekeeping RpoD (σ70).

RpoH protein family possesses a conserved region known as the 'RpoH box' and they also contain conserved sequences that are involved in the recognition of the -10 and -35 promoter elements (Martínez-Salazar et al., 2009; Nakahigashi et al., 1995; Wo, 1998).

Cellular response to heat shock is probably the most studied stress response bacteria although not extensively studied in rhizobia. Recent microarray data in *Sinorhizobium meliloti* indicated the upregulation of 169 genes in response to heat shock, including genes coding for chaperones and other heat shock proteins (Sauviac et al., 2007). Chaperone systems, such as DnaK–DnaJ and GroEL–GroES, are known as important components of the heat shock response. But they are also induced by other stressful conditions as well. These chaperones recognize exposed hydrophobic domains of denatured proteins and help to restore their original conformation. (Alexandre & Oliveira, 2011).

Nitrogen-fixing bacteria typically have multiple copies of *groEL* systems. *Bradyrhizobium japonicum* shows five *groESL* operons, which have different regulation systems and are differentially induced (Fischer et al., 1993). For example, groESL1,4,5 are heat inducible while groESL3 is induced by low-oxygen conditions (Babst et al., 1996; Fischer et al., 1993). The groESL1 is σ32 dependent, whereas the groESL4,5 is controlled by CIRCE element (Babst et al., 1996). In *Rhizobium leguminosarum*, only one of the three *groEL* homologues is needed for normal growth (Alexandre & Oliveira, 2011).

In rhizobia usually only one copy of *dnaK* is found. In contrast its co-chaperone *dnaJ* is found in more than one copies in the genome (Alexandre et al., 2008). In *B. japonicum*, *dnaK* plays a vital role than the *dnaJ* which was proven in an experiment done with knockout mutants. No *dnaK* mutants were obtained, while *dnaJ* mutants were successfully isolated, but with a reduced growth rate, especially at high temperatures (Minder et al., 1997). *groESL* and *dnaJ* may also

involve in the symbiotic performance, as already reported for several rhizobia species (Alexandre & Oliveira, 2011; Fischer et al., 1993; Labidi et al., 2000).

Table 2-1. List of bacterial strains and plasmids.

Strain or Plasmid	Genotype or phenotype	Reference
***E. coli* strain**		
DH5α	supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 F' (traD36, proAB+ lacIq, Δ(lacZ)M15) endA1 recA1 hsdR17 mcrA supE44λ gyrA96 relA1	Bethesda Research Laboratories
***Bradyrhizobium* sp.**		
B. japonicum USDA110	Cm^R Wild-type	USDA
Δ*blr7740* mutant	Cm^R Km^R *blr7740* :: Km	This work
Δ*blr2203* mutant	Cm^R Km^R *blr2203* :: Km	This work
Plasmids		
pKD4	Km^R expression vector	(Datsenko and Wanner 2000)
pKD78	Cm^R Lambda Red recombinase expression plasmid	(Datsenko and Wanner 2000)
pRK2013	Km^R cloning vector	(Ditta et al.1980)
pJQ200SK	Gm^R *sacB* suicide vector	(Quandt and Hynes 1993)
pJQ200SK- *blr7740*	Gm^R suicide vector containing *blr7740* gene	This work
pJQ200SK- *blr2203*	Gm^R suicide vector containing *blr2203* gene	This work
pKD78-pJQ200SK-*blr7740-λ*	Gm^R Cm^R suicide vector containing *blr7740* gene with Lambda Red recombinase expression plasmid	This work
pKD78-pJQ200SK-*blr2203-λ*	Gm^R Cm^R suicide vector containing *blr2203* gene with Lambda Red recombinase expression plasmid	This work
pKD78-pJQ200SK-*blr7740*-Km	Gm^R Km^R suicide vector containing *blr7740* gene substituted with kanamycin cassette	This work

pKD78-pJQ200SK-*blr2203*-Km	Gm^R Km^R suicide vector containing *blr2203* gene substituted with kanamycin cassette	This work

*Cm, Km and Gm refer to chloramphenicol, kanamycin, and gentamycin, respectively.

Table 2-2. Functional roles of significant genes responding to heat stress.

Functional groups	Heat shock treatment	
	Up	Down
Amino Acid Biosynthesis	14	13
Biosynthesis of cofactors, prosthetic groups, and carriers	18	9
Cell envelope	6	10
Cellular processes	33	37
Central intermediary metabolism	18	13
DNA replication, recombination, and repair	12	6
Energy metabolism	21	22
Fatty acid, phospholipid and sterol metabolism	13	10
Purines, pyrimidines, nucleosides, and nucleotides	7	7
Regulatory functions	49	27
Symbiosis	-	-
Transcription	8	5
Translation	23	15
Transport and binding proteins	44	29
Other categories	76	44
Hypothetical	279	268
Total	621	515

Table 2-3. Significantly up-regulated genes in response to heat shock at 42°C for 30 min.

Gene Name	Fold Change	Description
bll5218	35.6	hypothetical protein bll5218
blr7731	29.4	inositol monophosphatase family protein
bll7025	21.6	hypothetical protein bll7025
blr4499	18.6	hypothetical protein blr4499
bsr0071	18.1	hypothetical protein bsr0071
blr7740	18.0	small heat shock protein
bll4998	17.4	hypothetical protein bll4998
blr0072	16.5	hypothetical protein blr0072
blr1107	15.0	hypothetical protein blr1107
blr5221	14.8	small heat shock protein
blr7528	14.1	hypothetical protein blr7528
blr5227	13.8	heat shock protein
bsr1232	13.7	hypothetical protein bsr1232
bll5004	12.3	hypothetical protein bll5004
blr7979	12.3	monooxygenase
blr0680	11.9	chaperone protein
blr6174	11.2	ATP-dependent protease LA
blr2510	11.1	hypothetical protein blr2510
bll3015	11.0	putative Amicyanin precursor
blr1404	11.0	ATP-dependent protease ATP-binding subunit
blr7732	10.8	hypothetical protein blr7732
bsl4836	10.5	hypothetical protein bsl4836
blr2450	10.5	protease heat shock protein
bll3190	10.3	ABC transporter ATP-binding protein
blr2203	10.2	putative RNA polymerase sigma factor

Table 2-4. Significantly down-regulated genes in response to heat shock at 42°C for 30 min.

Gene *Name*	Fold change	Description
bll6866	-18.6	flagellin
bll7938	-15.3	hypothetical protein bll7938
blr0685	-12.4	dihydrodipicolinate reductase
blr7040	-11.1	cytochrome C-type protein
bll6865	-10.9	flagellin
bll4354	-10.9	hypothetical protein bll4354
blr4260	-10.6	hypothetical protein blr4260
blr7325	-10.2	hypothetical protein blr7325
bll2253	-10.2	hypothetical protein bll2253
bll6903	-10.1	outer membrane lipoprotein
blr7037	-9.8	periplasmic nitrate reductase
blr0639	-9.6	dephospho-CoA kinase
bsr2765	-8.5	cbb3 oxidase subunit IV
bll1804	-8.0	hypothetical protein bll1804
blr1373	-7.9	two-component hybrid sensor and regulator

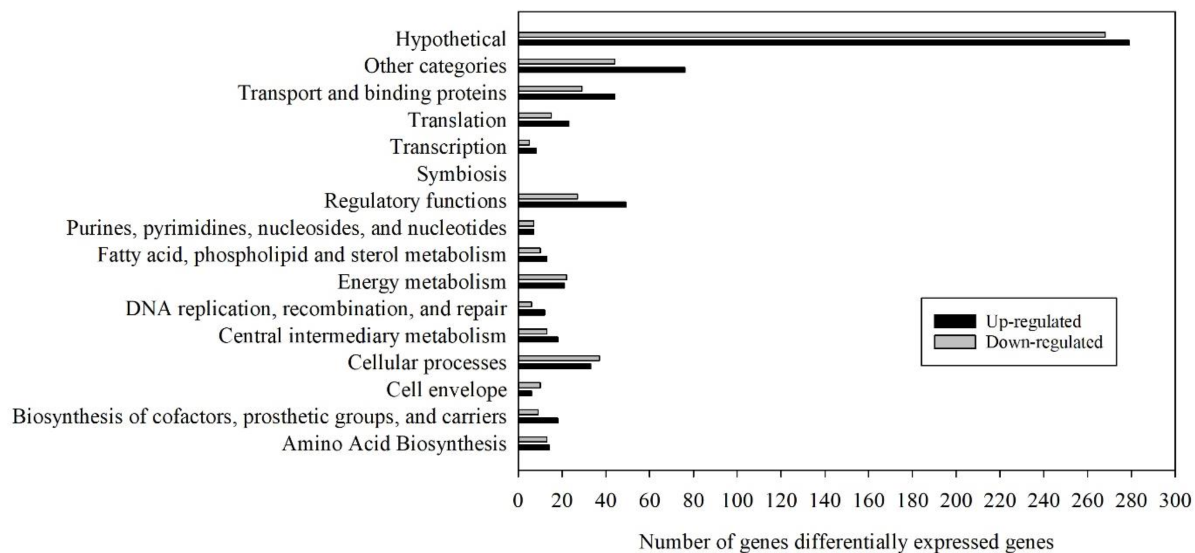

Figure 2- 2. Functional categories of statistically significant, differentially expressed genes under heat stress condition at 42°C for 30 min. Black bars, up-regulated genes; gray bars, down-regulated genes.

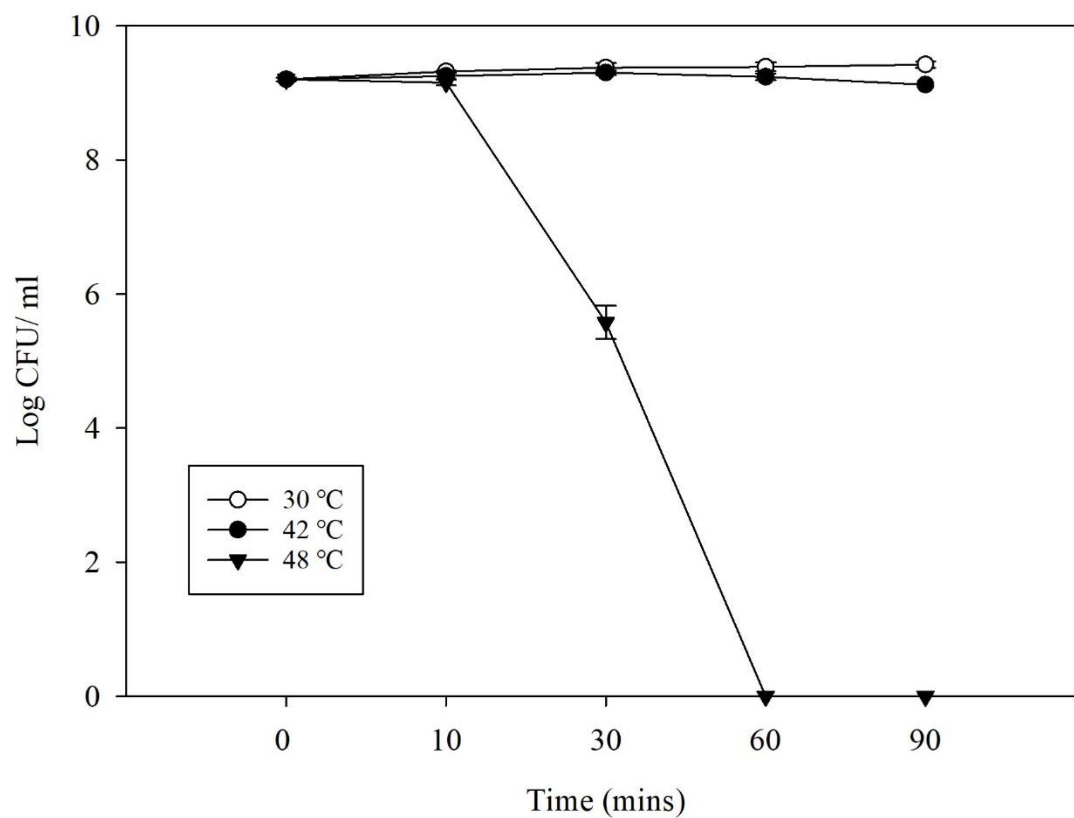

Figure 2- 3. Growth comparison of the wild type *B. japonicum* USDA110 under heat stress. Each time point represents the average log10 transformed CFU/ ml of three biological replicates. Error bars indicate the standard deviation of the means.

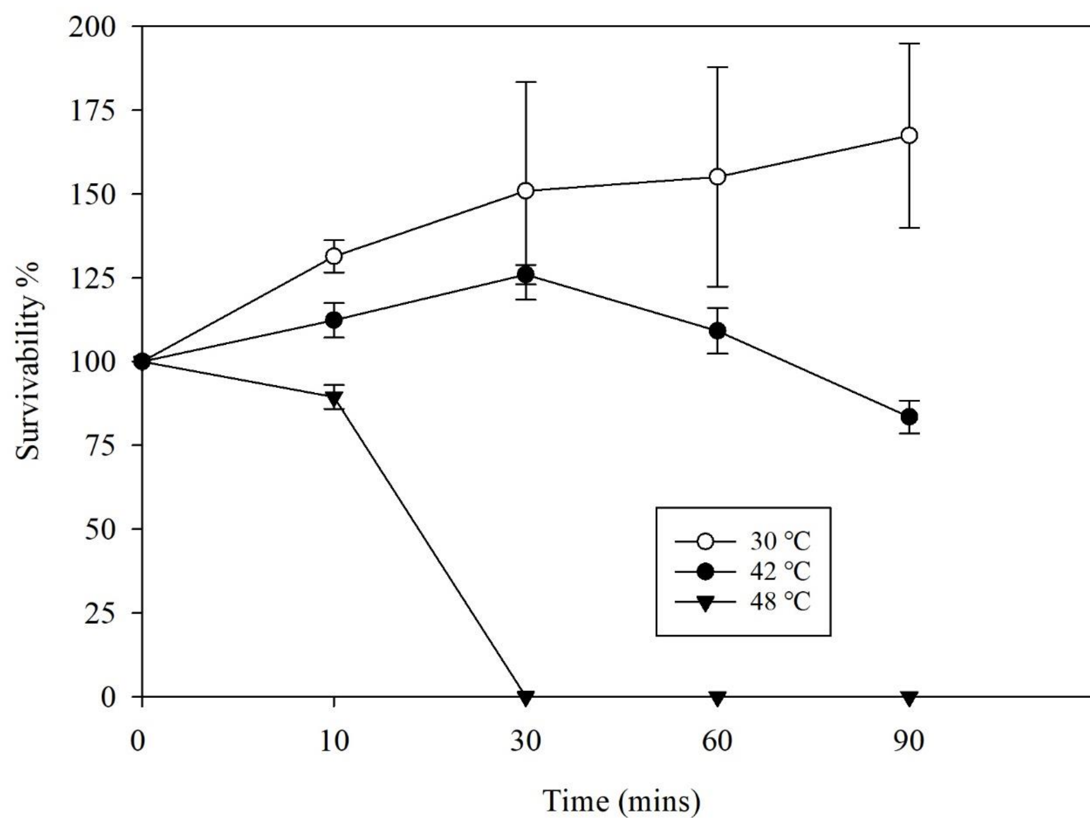

Figure 2- 4. Survivability% of the wild type *B. japonicum* USDA110 under heat stress. Each time point represents the average of three biological replicates. Error bars indicate the standard deviation of the means.

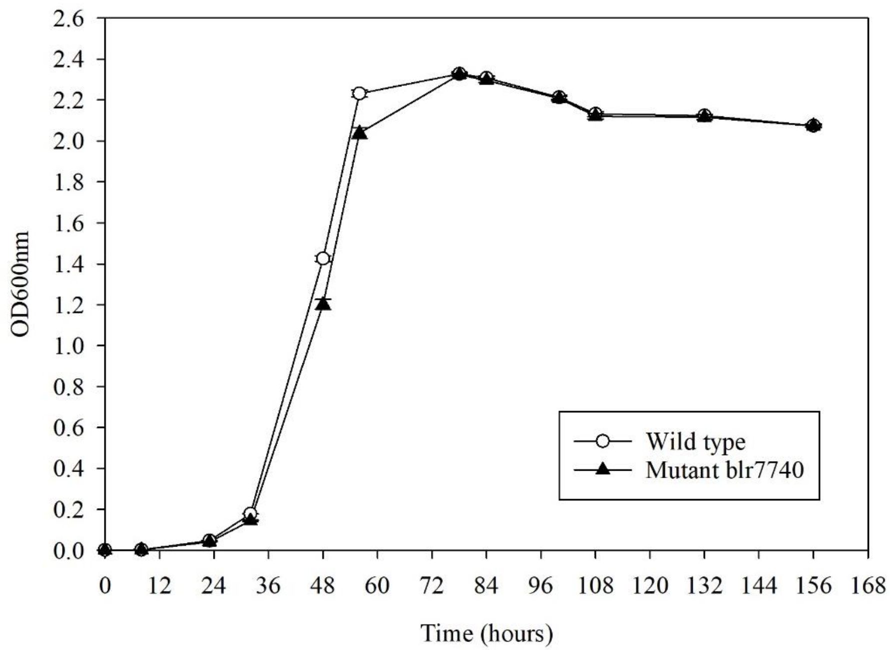

Figure 2- 5. Growth comparison of the wild type *B. japonicum* USDA110 and mutant *blr7740* under normal conditions. Each time point represents the average OD600 of three biological replicates. Error bars indicate the standard deviation of the means.

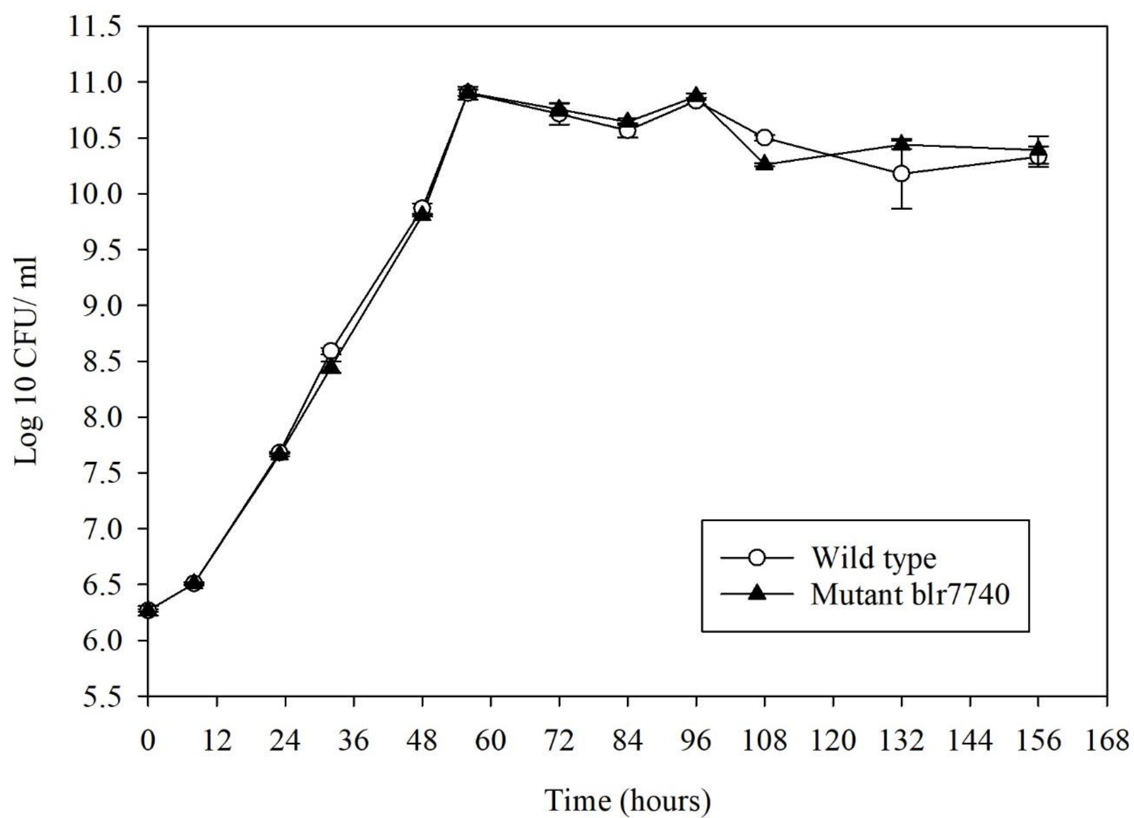

Figure 2- 6. Growth comparison of the wild type *B. japonicum* USDA110 and mutant *blr7740* under normal conditions. Each time point represents the average log10 transformed CFU/ml three biological replicates. Error bars indicate the standard deviation of the means.

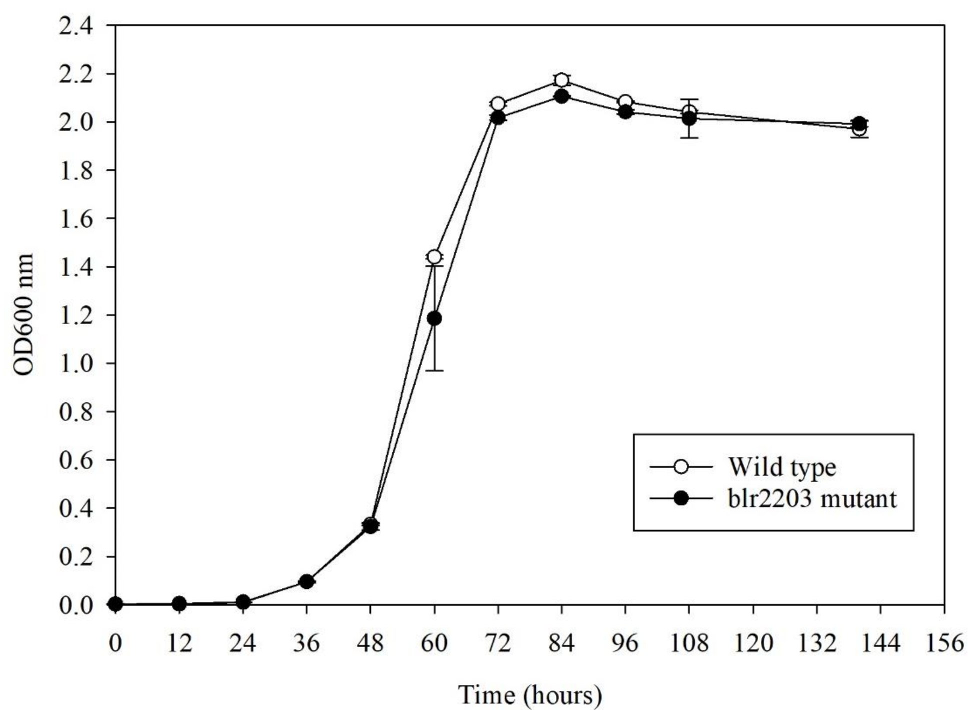

Figure 2- 7. Growth comparison of the wild type *B. japonicum* USDA110 and mutant *blr2203* under normal conditions. Each time point represents the average OD600 of three biological replicates. Error bars indicate the standard deviation of the means.

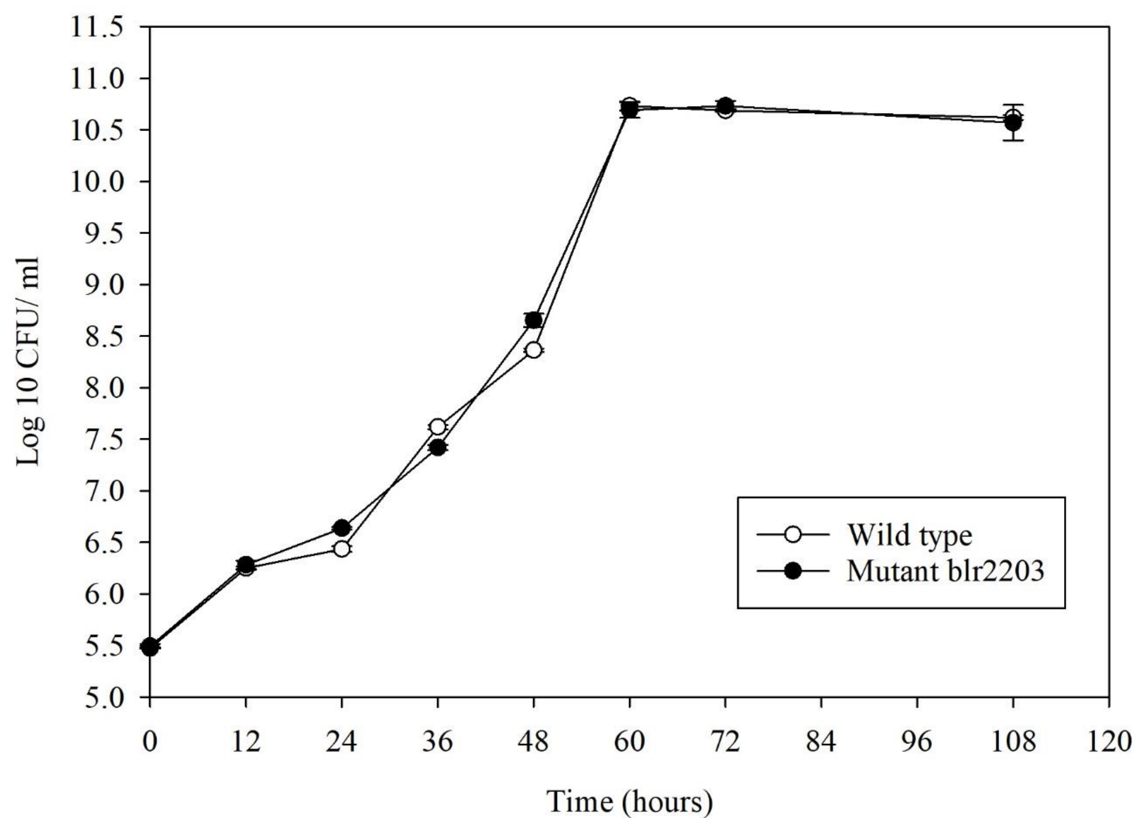

Figure 2- 8. Growth comparison of the wild type *B. japonicum* USDA110 and mutant *blr2203* under normal conditions. Each time point represents the average log10 transformed CFU/ml three biological replicates. Error bars indicate the standard deviation of the means.

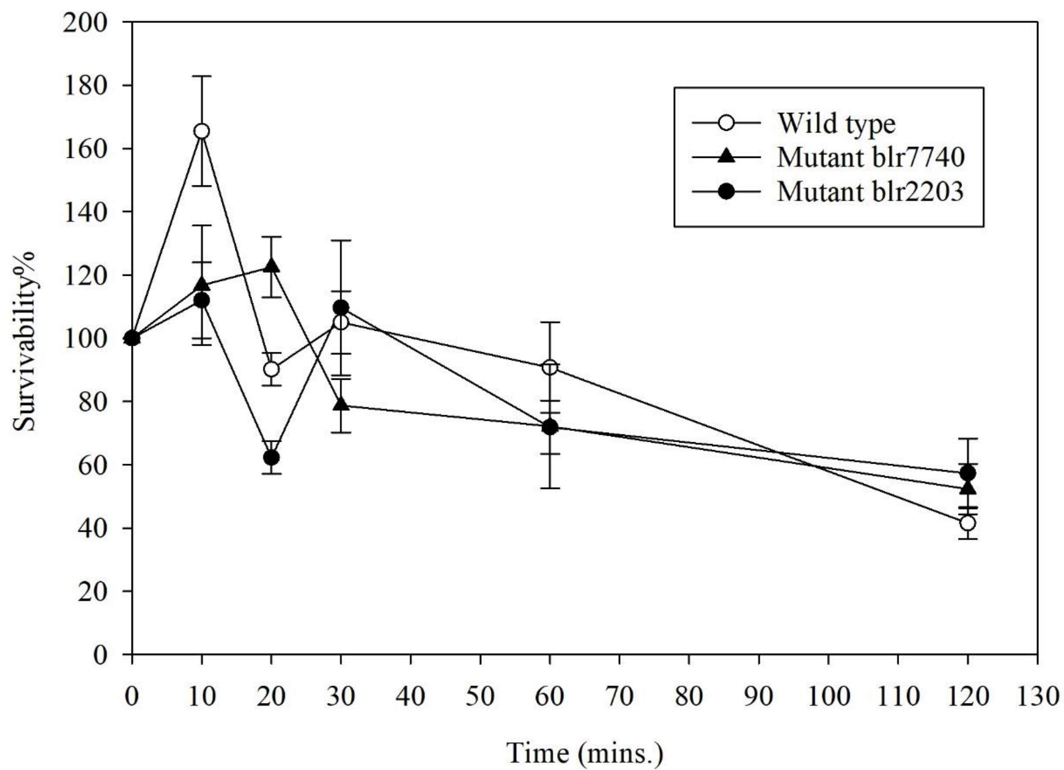

Figure 2- 9. Survivability% of the wild type *B. japonicum* USDA110, mutant *blr7740* and mutant *blr2203* under heat stress. Each time point represents the average of three biological replicates. Error bars indicate the standard deviation of the means.

REREFENCES

Alexandre, A., Laranjo, M., Young, J. P. W., & Oliveira, S. 2008. DnaJ is a useful phylogenetic marker for alphaproteobacteria. *Int. J. Syst. Evol. Microbiol.*, **58**, 2839–2849.

Alexandre, A., & Oliveira, S. 2011. Most heat-tolerant rhizobia show high induction of major chaperone genes upon stress. *FEMS Microbiol. Ecol.*, **75**, 28–36.

Arsène, F., Tomoyasu, T., & Bukau, B. 2000. The heat shock response of *Escherichia coli*. *Int. J. Food Microbiol.*, **55**, 3–9.

Babst, M., Hennecke, H., & Fischer, H. M. 1996. Two different mechanisms are involved in the heat-shock regulation of chaperonin gene expression in *Bradyrhizobium japonicum. Mol. Microbiol.*, **19**, 827–839.

Bertani, G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.*, **62**, 293–300.

Bohne, J., Sokolovic, Z., & Goebel, W. 1994. Transcriptional regulation of prfA and PrfA-regulated virulence genes in *Listeria monocytogenes*. *Mol. Microbiol.*, **11**, 1141–1150.

Bushby, H. V. A., & Marshall, K. C. 1977. Some factors affecting the survival of root-nodule bacteria on desiccation. *Soil Biol. Biochem.*, **9**, 143–147.

Chang, W.-S., Franck, W. L., Cytryn, E., Jeong, S., Joshi, T., Emerich, D. W., Sadowsky, M. J., Xu, D., & Stacey, G. 2007. An Oligonucleotide Microarray Resource for Transcriptional Profiling of *Bradyrhizobium japonicum*. *Mol. Plant-Microbe Interact. MPMI*, **20**, 1298–1307.

Datsenko, K. A., & Wanner, B. L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.*, **97**, 6640–6645.

Delory, M., Hallez, R., Letesson, J. J., & De Bolle, X. 2006. An RpoH-like heat shock sigma factor is involved in stress response and virulence in *Brucella melitensis* 16M. *J. Bacteriol.*, **188**, 7707–7710.

Ditta, G., Stanfield, S., Corbin, D., & Helinski, D. 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of Rhizobium meliloti. Proc. Natl. Acad. Sci. **77**(1): 7347-7351.

Diouf, A., De Lajudie, P., Neyra, M., Kersters, K., Gillis, M., Martinez-Romero, E., & Gueye, M. 2000. Polyphasic characterization of rhizobia that nodulate *Phaseolus vulgaris* in West Africa (Senegal and Gambia). *Int. J. Syst. Evol. Microbiol.*, **50**, 159–170.

Fischer, H. M., Babst, R., Kaspar, T., Acuna, G., Arigoni, F., & Hennecke, H. 1993. One member of a groESL-like chaperonin multigene family in *Bradyrhizobium japonicum* is co-regulated with symbiotic nitrogen fixation genes. *EMBO J.*, **12**, 2901–2912.

Galibert, F., Finan, T. M., Long, S. R., Pühler, A., Abola, P., Ampe, F., Barloy-Hubler, F., Barnet, M. J., Becker, A., Boistard, P., Bothe, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R. W., … Batut, J. 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science.* **293**, 668–672.

González, V., Santamaría, R. I., Bustos, P., Hernández-González, I., Medrano-Soto, A., Moreno-Hagelsieb, G., Janga, S. C., Ramírez, M. A., Jiménez-Jacinto, V., Collado-Vides, J., & Dávila, G. 2006. The partitioned *Rhizobium etli* genome: Genetic and metabolic redundancy in seven interacting replicons. *Proc. Natl. Acad. Sci. U. S. A.*, **103**,

3834–3839.

Green, H. A., & Donohue, T. J. 2006. Activity of *Rhodobacter sphaeroides* RpoHII, a second member of the heat shock sigma factor family. *J. Bacteriol.*, **188**, 5712–5721.

Guisbert, E., Herman, C., Lu, C. Z., & Gross, C. A. 2004. A chaperone network controls the heat shock response in *E. coli*. *Genes Dev.*, **18**, 2812–2821.

Jeon, Jeong-Min, Lee, Hae-In, Donati, Andrew J, So, Seon-Jae, Emerich, David W, Chang, W.-S. 2011. Whole-Genome Expression Profiling of *Bradyrhizobium japonicum* in Response to Hydrogen Peroxide. *MPMI*, **24**, 1472–1481.

Kaneko, T., Nakamura, Y., Sato, S., Asamizu, E., Kato, T., Sasamoto, S., Watanabe, A., Idesawa, K., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Mochizuki, Y., Nakayama, S., Nakazaki, N., … Tabata, S. 2000. Complete Genome Structure of the Nitrogen-fixing Symbiotic Bacterium *Mesorhizobium loti. DNA Res.,* **7**, 331–338.

Kaneko, T., Nakamura, Y., Sato, S., Minamisawa, K., Uchiumi, T., Sasamoto, S., Watanabe, A., Idesawa, K., Iriguchi, M., Kawashima, K., Kohara, M., Matsumoto, M., Shimpo, S., Tsuruoka, H., Wada, T., Yamada, M., & Tabata, S. 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. *DNA Res.*, **9**, 189–197.

Karanja, N. K., & Wood, M. 1988. Selecting *Rhizobium phaseoli* strains for use with beans (*Phaseolus vulgaris* L.) in Kenya: Infectiveness and tolerance of acidity and aluminium. *Plant Soil*, **112**, 7–13.

Labidi, M., Laberge, S., Vezina, L. P., & Antoun, H. 2000. The dnaJ (hsp40) locus in *Rhizobium leguminosarum bv. phaseoli* is required for the establishment of an effective symbiosis with *Phaseolus vulgaris*. *Mol. Plant-Microbe Interact.*, **13**, 1271–1274.

Lynch, D. H., & Smith, D. L. 1994. The effects of low root-zone temperature stress on two soybean (*Glycine max*) genotypes when combined with Bradyrhizobium strains of varying geographic origin. *Physiol. Plant.*, **90**, 105–113.

Martínez-Salazar, J. M., Sandoval-Calderón, M., Guo, X., Castillo-Ramírez, S., Reyes, A., Loza, M. G., Rivera, J., Alvarado-Affantranger, X., Sánchez, F., González, V., Dávila, G., & Ramírez-Romero, M. A. 2009. The *Rhizobium etli* RpoH1 and RpoH2 sigma factors are involved in different stress responses. *Microbiology*, **155**, 386–397.

Matuszewska, M., Kuczyńska-Wiśnik, D., Laskowska, E., & Liberek, K. 2005. The small heat shock protein IbpA of *Escherichia coli* cooperates with IbpB in stabilization of thermally aggregated proteins in a disaggregation competent state. *J. Biol. Chem.*, **280**, 12292–12298.

Michiels, J., Verreth, C., & Vanderleyden, J. 1994. Effects of temperature stress on bean-nodulating Rhizobium strains. *Appl. Environ. Microbiol.*, **60**, 1206–1212.

Minder, A. C., Fischer, H. M., Hennecke, H., & Narberhaus, F. 2000. Role of HrcA and CIRCE in the heat shock regulatory network of *Bradyrhizobium japonicum*. *J. Bacteriol.*, **182**, 14–22.

Minder, A. C., Narberhaus, F., Babst, M., Hennecke, H., & Fischer, H. M. 1997. The dnaKJ operon belongs to the σ32-dependent class of heat shock genes in *Bradyrhizobium japonicum. Mol. Gen. Genet.*, **254**, 195–206.

Missiakas, D., Betton, J. M., & Raina, S. 1996. New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA and Skp/OmpH. *Mol. Microbiol.*, **21**, 871–884.

Missiakas, D., & Raina, S. 1997. Protein misfolding in the cell envelope of *Escherichia coli*: New signaling pathways. *Trends Biochem. Sci.*, **22**, 59–63.

Mogk, A., Schlieker, C., Friedrich, K. L., Schönfeld, H. J., Vierling, E., & Bukau, B. 2003. Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK. *J. Biol. Chem.*, **278**, 31033–31042.

Mogk, A., Tomoyasu, T., Goloubinoff, P., Rüdiger, S., Röder, D., Langen, H., & Bukau, B. 1999. Identification of thermolabile *Escherichia coli* proteins: Prevention and reversion of aggregation by DnaK and ClpB. *EMBO J.*, **18**, 6934–6949.

Munévar, F., & Wollum, A. G. 1981. Growth of *Rhizobium japonicum* Strains at Temperatures Above 27°C. *Appl. Environ. Microbiol.*, **42**, 272–276.

Nakahigashi, K., Yanagi, H., & Yura, T. 1995. Isolation and sequence analysis of rpoH genes encoding σ32 homologs from gram negative bacteria: Conserved mRNA and protein segments for heat shock regulation. *Nucleic Acids Res.*, **23**, 4383–4390.

Narberhaus, F. 1999. Negative regulation of bacterial heat shock genes. *Mol. Microbiol.*, **31**, 1–8.

Narberhaus, F., Käser, R., Nocker, A., & Hennecke, H. 1998. A novel DNA element that controls bacterial heat shock gene expression. *Mol. Microbiol.*, **28**, 315–323.

Narberhaus, F., Krummenacher, P., Fischer, H. M., & Hennecke, H. 1997. Three disparately

regulated genes for σ32-like transcription factors in *Bradyrhizobium japonicum*. *Mol. Microbiol.*, **24**, 93–104.

Nour, S. M., Fernandez, M. P., Normand, P., & Cleyet-Marel, J. C. 1994. *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). *Int. J. Syst. Bacteriol.*, **44**, 511–522.

Quandt, J., & Hynes, M. F. 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. *Gene*, **127**, 15–21.

Roncarati, D., & Scarlato, V. 2017. Regulation of heat-shock genes in bacteria: from signal sensing to gene expression output. *FEMS Microbiol. Rev.*, **41**, 549–574.

Sadowsky, M. J., Tully, R. E., Cregan, P. B., & Keyser, H. H. 1987. Genetic Diversity in *Bradyrhizobium japonicum* Serogroup 123 and Its Relation to Genotype-Specific Nodulation of Soybean. *Appl. Environ. Microbiol.*, **53**, 2624–2630.

Sauviac, L., Philippe, H., Phok, K., & Bruand, C. 2007. An extracytoplasmic function sigma factor acts as a general stress response regulator in *Sinorhizobium meliloti*. *J. Bacteriol.*, **189**, 4204–4216.

Somasegaran, P., Hoben, H. J., Somasegaran, P., & Hoben, H. J. 1994. Quantifying the Growth of Rhizobia. *Handb. Rhizobia*, 47–57.

Straus, D. B., Walter, W. A., & Gross, C. A. 1987. The heat shock response of *E. coli* is regulated by changes in the concentration of σ32. *Nature*, **329**, 348–351.

Street, T. O., Zeng, X., Pellarin, R., Bonomi, M., Sali, A., Kelly, M. J. S., Chu, F., & Agard,

D. A. 2014. Elucidating the mechanism of substrate recognition by the bacterial Hsp90 molecular chaperone. *J. Mol. Biol.*, **426**, 2393–2404.

Tusher, V. G., Tibshirani, R., & Chu, G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. U. S. A.*, **98**, 5116–5121.

Veinger, L., Diamant, S., Buchner, J., & Goloubinoff, P. 1998. The small heat-shock protein IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *J. Biol. Chem.*, **273**, 11032–11037.

Wawrzynow, A., Banecki, B., & Zylicz, M. 1996. The Clp ATPase define a novel class of molecular chaperones. *Mol. Microbiol.*, **21**, 895–899.

Weidmann, S., Maitre, M., Laurent, J., Coucheney, F., Rieu, A., & Guzzo, J. 2017. Production of the small heat shock protein Lo18 from *Oenococcus oeni* in *Lactococcus lactis* improves its stress tolerance. *Int. J. Food Microbiol.*, **247**, 18–23.

Wo, M. M. S. M. 1998. Eubacterial sigma-factors. *FEMS Microbiol. Rev*. **22**, 127-150.

Xu, Z., Horwich, A. L., & Sigler, P. B. 1997. *The Crystal Structure of the asymmetric GroEL-GroES-(ADP)7 chaperonin complex*. **1303**, 1299–1303.

Zhang, F., Dashti, N., Hynes, R. K., & Smith, D. L. 1996. Plant growth promoting rhizobacteria and soybean [*Glycine max* (L.) Merr.] nodulation and nitrogen fixation at suboptimal root zone temperatures. *Ann. Bot.*, **77**, 453–460.

CHAPTER 3

PHYSIOLOGICAL RESPONSES OF THE SOYBEAN SYMBIONT *BRADYRHIZOBIUM JAPONICUM* TO ACID SHOCK

ABSTRACT

Soil pH is one of the most influential environmental factors that regulate growth and function of soil bacteria. Extreme pH levels beyond the optimum range have deleterious effects on cell viability and symbiotic functions of rhizobia. Acidification of soil is a natural process which is expedited in agricultural soils due to long term agronomic practices and root exudates of certain crops, therefore, studying and identifying the acid shock responses at the cellular and molecular level is imperative at developing a successful inoculant for field application. Although *Bradyrhizobium japonicum* is a conventional N biofertilizer that is being used in soybean fields for decades, there is still much room for improvements of its performance in the soil. In this study, genetics and physiology of *B. japonicum* was investigated under acid shock using genome wide transcriptional profiling. Growth curve analysis conducted under different pH conditions showed that exposure to ca. pH 4 (i.e., 500 µl of 2N HCl in 100 ml) for 4 h would be the stressful condition under which the cells can still grow even at a slow rate. Whole genome transcriptional profiling under this condition using microarray technology showed that 582 and 619 genes were up and down regulated in response to acid shock, respectively. Multi-drug resistance efflux pumps and chaperones were prominent among the up-regulated genes and more genes related to chemotaxis and cell division were suppressed. Mutagenesis studies performed on highly up-regulated genes under acid shock showed that multi-drug resistance efflux pump coding gene *blr7593* plays a vital role under acid shock while both *blr7593* and VirG like two component response regulator protein coding genes are not crucial for normal growth.

INTRODUCTION

Soil acidity is one of the major constraints in agriculture including legume production. In general, acidic soils accompanying high Al^{3+} toxicity and low P availability affect the growth of both host and symbiotic partner. Soil acidity is also associated with low levels of potassium and other micronutrients such as calcium, magnesium and molybdenum which are essential for growth and functioning of both plants and microorganisms (Puranamaneewiwat et al., 2006). In addition, soil acidity challenges SNF between rhizobia and their host plant in many ways. Specifically, the microsymbiont is more prone to acidity than the host plant (O'Hara & Glenn, 1994). Acidic soils limit rhizobial nodulation ultimately affecting the efficiency of SNF because of its detrimental effects on initial stages of the symbiosis.

Soil pH is directly responsible for the growth, persistence, multiplication, and establishment of symbiosis by rhizobia. The host plant itself is also vulnerable to soil acidity, but the impact on rhizobia is more detrimental. Failure to survive and persist in soil will affect their competitiveness and efficiency in SNF. Inconsistency of in-vitro and in-vivo performances leads to ultimate refusal of the inoculum from the industry due to dissatisfaction of commercial growers. Therefore, understanding the robustness of inoculants under field conditions is the key step to produce a successful N-biofertilizer. In addition to endurance and proliferation of rhizobia, initiation of symbiosis is also distracted by soil acidity. Acidic pH disrupts the signal exchange between the host plant and the symbiont at early stages of nodulation (Hungria & Vargas, 2000). Flavonoids secretion by host plants is reduced under acidic conditions, affecting the excretion of Nod factors due to decreased expression of *nod* genes in rhizobia. The reduced Nod factor signaling retards the subsequent root hair deformation and curling. Additionally, Ca^{2+} deficit acidic soils impair Ca^{2+} mediated root hair attachment of rhizobia and following root colonization (Lin

et al., 2020). That causes a detrimental effect on the formation of infection thread and invasion of the plant root by the microsymbiont. Also, low pH severely hinders the expression of early nodulation genes, initial cell division and nodule primordia formation. This has caused a remarkable reduction of nodule numbers, nodule dry weight and functionality of nodules (Lie, 1969; Lin et al., 2020). Furthermore, soil acidity associated molybdenum scarcity results in reduced nitrogenase activity and nitrogen fixation as molybdenum is an integral component of nitrogenase enzyme.

A multitude of root nodulating bacteria that exhibit high levels of survival, growth and symbiotic performances in low pH soils have been identified in previous laboratory and field experiments (Aarons, 1991). A variety of rhizobial species have been isolated and identified from root nodules of legumes grown in acidic soils worldwide. However, the relationship between soil acidity and competitiveness of rhizobia is complicated. For example, rhizobia recovered from acidic soils are conversely sensitive to acidity. It is proposed that these rhizobia are protected from extreme acidity by soil micro niches. Therefore, no guarantee is provided that the rhizobial strains isolated from acidic soils will be acid-tolerant when it is used as an inoculant in a low pH soil (Rhichardson & Simpson, 1989). Degree of tolerance, survival, growth, and symbiotic potency in response to low soil pH highly varies depending on the rhizobial strain. In general, *Snorhizobium fredii* is known to be more competitive than *B. japonicum* in nodulating soybeans in neutral soils, and in contrast *B. japonicum* dominated *S. fredii* in soil at pH 4.9 (Triplett & Sadowsky, 1992). Additionally, more competitive native rhizobial strains can be dominated by introduced strains under sudden acidification of soils. For instance, it is reported that *R. etli* which is more competitive in bean nodulation than *R. tropici* was replaced by introduced acid tolerant *R. tropici* after abrupt acidification of soil (Anyngo et al., 1995; Sadowsky, 2006). Therefore, the behavioral

changes of rhizobia in response to soil pH fluctuations are highly unforeseen. Slow growing rhizobia such as *Bradyrhizobium* spp. are reported to be more acid tolerant than fast growing *Rhizobium* spp. (Ferguson et al., 2013). *B. japonicum* is one of the most acid tolerance and effective species among rhizobia (Puranamaneewiwat et al., 2006). However, their growth and existence are highly diverse. Combined effect of acidity, P deficiency and Al toxicity restricts the multiplication of *Bradyrhyzobium* spp. (Keyser & Munns, 1979). A laboratory experiment performed in acidic soils with *Bradyrhizobium* strains PSR007, PSR011, ASR002 and ASR003 was evident high survival, growth, and symbiotic performances of *Bradyrhizobium* spp. at low pH levels (Appunu & Dhar, 2006). A study conducted with five strains of *B. japonicum* and seven soybean cultivars for their tolerance and symbiosis under acidity (pH 4.2, 4.4 and 4.6) and presence of Al under low P availability revealed that strains USDA 110 and 6 are more tolerant than USDA 122, 138 and 143. However, the significant reduction of cell numbers showed by even more tolerance strains in this experiment suggested that colonization on soybean roots and nodulation by rhizobia are negatively affected by these stressful conditions in the field directly (Taylor et al., 1991). Additionally, more rhizobial species have been discovered to be acid-tolerant and effective under low soil pH associated inhospitable soils. *Rhizobium tropici* CIAT899 is a nodule-forming α-proteobacterium in common bean (*Phaseolous vulgaris*) known to possess an intrinsic resistance to abiotic stressful conditions including low pH. (Martinez-Romero et al., 1991; Strous et al., 2018). Furthermore, *R. trifolii* was reported to show different levels of tolerance to soil acidity in their symbiosis with the host (Taylor et al., 1991).

Soil acidity can affect the growth and survival of rhizobia in several different ways. Apart from the direct effect of elevated H^+ concentrations on cellular activities, increased solubility of metals such as Al, Cu, and Mn also inhibit the growth and persistence of rhizobia. Moreover, soil

Ca and P levels are also regulated by soil pH and subsequently affect the growth of rhizobia. However, several studies show that the acid tolerance of rhizobia is correlated with the strains ability to maintain a neutral pH (7.2-7.5) inside the cells, which can be achieved by proton exclusion, enhanced buffering capacity of cytoplasm, acid shock responses, presence of glutathione, elevated potassium and glutamate concentrations, changes in membrane permeability, and calcium metabolism. (Graham et al., 1994; Kashket, 1985; O'Hara et al., 1989).

Microsymbionts such as rhizobia are severely affected by acidic environments in both free living and symbiotic stages. During the transition from free living to symbiotic stage, sudden decline of pH to extremely low levels could be challenging and require rapid responses and adaptations. Recent advancements in genomics, transcriptomics, and proteomics analysis have enabled the scientists to unveil genetic basis of rhizobial acid responses at the cellular level. The biggest problem with inoculant strains is that correlating *in-vitro* growth on acidic media with the field performance of root nodule bacteria.

There has been little success in correlating bacterial growth on acidic media with the field performance of an inoculant (Bromfield & Jones, 1980; Keyser et al., 1979; Rice et al., 1982). The capacity to maintain intracellularly neutral pH under acidic conditions, has been proposed as an essential mechanism for growth and survival of rhizobia (O'Hara et al., 1989). However, there might be numerous other mechanisms that involve in pH shock response by rhizobia. In this study we are focusing on the cellular responses of *B. japonicum* USDA110 towards acidic conditions using whole genome transcriptomic profiling. The aim of this study is to identify significantly regulated genes in response to acid shock and to evaluate possible means of improving symbiotic performances of the bacterium in the field.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 3-1. The wild type strain of *Bradyrhizobium japonicum* USDA110 was cultured in arabinose-gluconate (AG) medium at pH 6.8 which contains 125 mg of Na_2HPO_4, 250 mg of Na_2SO_4, 320 mg of NH_4Cl, 180 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2$, 4 mg of $FeCl_3$, 1.3 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1g of 2-(N-morpholino) ethanesulfonic acid (MES), 1 g of yeast extract, 1 g of L-arabinose, and 1 g of D-gluconic acid sodium sulfate per L of distilled water (Sadowsky et al., 1987). *Bradyrhizobium japonicum* USDA110 was incubated aerobically at 30°C with shaking at 200 rpm when grown in liquid media. Antibiotics used for strain selection were chloramphenicol (50 μgml^{-1} for wild type strain of *Bradyrhizobium japonicum* USDA110 and mutants) and kanamycin (150 μgml^{-1} for mutant strains). *Escherichia coli* strains and plasmids were grown aerobically in Luria-Bertani (LB) medium at pH 7.0 which contains 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of distilled water (Bertani, 1951). All *Escherichia coli* cultures were grown at 37°C (except DH5α pKD78 incubated at 30°C), with shaking at 200 rpm overnight in liquid media. Appropriate antibiotics were added into media when needed as follows: gentamicin (15 μgml^{-1} for pJQ200SK), chloramphenicol (15 $\mu gml^{-1}$for pKD78) and kanamycin (50 μgml^{-1} for pRK2013 and pKD4). When preparing agar plates of each medium, 15 g of agar was added per L of distilled water.

Acid shock assay. *B. japonicum* USDA110 cultures were grown in AG media at 30℃ aerobically with vigorous shaking at 200 rpm until mid-log phase. Subcultures of 500 ml each with chloramphenicol were grown at 30℃ aerobically with shaking at 200 rpm until reached exponential phase with OD_{600nm} of 0.8-1.0. Culture was mixed well and divided into 100 ml each in 250 ml flasks for pH shock treatment. To provide the pH shock condition to bacteria 500 µl of

HCl with different concentrations (1N, 2N, 4N, 6N, 8N, 10N and 12.1N) was added to 100 ml of AG culture media. Five hundred microliters of autoclaved distilled water were added into the control sample. Then the cultures were incubated for different time intervals (4, 8 and 16 h) at 30°C with shaking at 200 rpm. Each treatment was replicated three times. OD_{600nm}, pH and CFU/ ml were measured before and after the pH shock treatment.

RNA isolation. Total RNA was isolated from *B. japonicum* USDA110 cultures treated with 2N HCl for 4 h. 200 ml of *B. japonicum* USDA110 culture was grown at 30°C with shaking at 200 rpm until it reaches OD 600_{nm} 0.8-1.0. Then 500 µl of 2N HCl was added to 100 ml of the AG media culture and was incubated at 30°C for 4 h under acid shock. Five hundred microliters of sterilized distilled water were added to the remaining 100 ml of culture which was maintained at 30°C as the control group. The experiment was conducted in three biological replicates. RNA extraction was performed using hot phenol method as previously described (Jeon et al., 2011). Cells were harvested by centrifugation for 20 min at 4°C and 4,000 rpm in a fixed angle rotor. Then the cell pellet was stored at -80°C until use after decanting the supernatant. The RNeasy mini kit (Qiagen) and RNase-free DNase (Qiagen) was used to purify the isolated RNA according to manufacturer's protocol. RNA concentration was measured on NanoDrop (Thermo Scientific) and RNA quality was checked using gel electrophoresis.

Gene expression analysis by microarray hybridization. Whole-Genome transcriptional profiles of *B. japonicum* USDA110 in response to acid shock were created by the hybridization of cDNA samples labeled with Cy3 and Cy5 dyes (GE Healthcare) to microarray chips containing 70-mer oligonucleotides that were complementary to each of the 8,453 annotated open reading frames (ORFs) of *B. japonicum* (Chang et al., 2007). Thirty micrograms of total RNA were used for cDNA synthesis, and 5 µg of cDNA from both control and experimental conditions was used

for labeling and hybridization. cDNA synthesis, cDNA labeling, hybridization, and washing was done as described previously (Chang et al., 2007). Three independent biological replicates were prepared for each condition, with a dye-swap for each replicate, resulting a total of 6 slides for each experimental condition.

Statistical analysis of microarray data. The slides were scanned with the Axon GenePix 4200 scanner, and GenePix Pro 6.0 software was used to measure intensity values at each spot. The signal intensities were normalized for slide and spot abnormalities using the locally weighted scatterplot smoothing (LOWESS) algorithm and subsequently analyzed by mixed-effect microarray analysis of variance (MAANOVA) (Jeon et al., 2011). Values obtained from this round of analysis were input into a significance analysis of microarray (SAM) statistical package (Tusher et al., 2001) to create a list of differentially expressed genes with a fold induction threshold of 1.5 or 2.0 and a false-discovery rate (FDR) of 5% or less ($q <= 0.05$ [q is the adjusted P value by FDR-based multiple testing correction])

qRT-PCR analysis. Genes which were significantly expressed in the microarray experiment were chosen for quantitative reverse transcription-PCR (qRT-PCR) analysis to confirm the microarray data (Table 3-2). Primers were designed with Primer 3 software (http://frodo.wi.mit.edu/primer3/) to amplify 80- to 250-bp regions of the chosen genes (Table 3-3). Two micrograms of the same RNA that was used in the microarray experiment was employed to synthesize cDNA. The process was performed according to a previously described protocol (Cytryn et al., 2007). Relative expression values for three biological replicates were normalized to the expression values of a housekeeping gene (*bll0631*; parA), which encodes a chromosome-partitioning protein. Fold induction values were calculated in accordance with the method of Pfaffl (Pfaffl, 2001).

Construction of mutant strains. Mutant strain was constructed using site-specific mutagenesis to delete the selected genes (*blr2694* and *blr7593*) that was upregulated in response to acid shock, from the genome of *B. japonicum* USDA110. A 1.81 kb fragment containing the *B. japonicum* USDA110, VirG like two component response regulator coding gene *blr2694*, was amplified by PCR using forward primer *blr2694*_FW (5' ATATGGATCCACCTCGGTATCGTCAAAGA 3') and reverse primer *blr2694*_RV (5' ATATAGGATCCAACGCGGCACCATAAAG 3') which have restriction enzyme recognition linker for BamHI at 5' end of each primer.

The *B. japonicum* USDA110, multidrug resistance efflux pump coding gene under acid shock, blr7593, was amplified as a 1.81kb long DNA fragment using forward primer *blr7593*_FW (5' ATATAGGATCCGGTCGACATGTTGAGCTTG 3') and reverse primer *blr7593*_RV (5' ATATAGGGCCCCGTTGGTGAGGAGGTAGAT 3') which have restriction enzyme recognition linkers for BamHI (*blr7593*_FW) and ApaI (*blr7593*_RV) respectively at 5' ends. PCR amplification was performed on C1000 Thermo Cycler (Biorad, Hercules, CA) followed by gel electrophoresis, using a PowerPacTM Basic (Biorad, Hercules, CA), to confirm the proper amplification of the target regions. Then, the target amplicon was inserted into the pJQ200SK (gentamycin resistant) suicide vector plasmid (Quandt & Hynes, 1993) through restriction digestion by the restriction enzyme BamHI followed by ligation. The recombinant pJQ200SK vector plasmid carrying the gene of interest was then introduced into DH5α competent cells via heat shock transformation. The vector plasmid with the clone were transformed into electrocompetent DH5α pKD78 (chloramphenicol resistance) cells carrying the Lambda Red Recombinase expression plasmid pKD78, via electroporation with Micro PulserTM (Biorad, Hercules, CA), at 1.8 kV. Kanamycin resistance cassette (1.4kb) was amplified from pKD4

(kanamycin resistance) plasmid by PCR using the forward primer (PS1): 5’ GTGTAGGCTGGAGCTGCTTC 3’ and reverse primer (PS2): 5’ CATATGAATATCCTCCTTAG 3’ (Datsenko & Wanner, 2000). Then the kanamycin cassette was re-amplified with two 60-bp primers, which have 40-bp linkers homologous to both bordering regions of the deletion target at 5’ ends of PS1 and PS2 using Phusion Taq polymerase enzyme.

For *blr2694* gene FW_60 bp primer: 5’ACGTGACCCGGTTGGTGACGACGGATTTTGAGAGTGAAGTGTGTAGGCTGGAGCTGCTTC 3’ and RV_60 bp primer: 5’ CACTTCGCGCTAACGTGACCCTTCGGGTCCGGATCTTGCCCATATGAATATCCTCCTTAG 3’ were used. For *blr7593* gene FW_60 bp primer: 5’ GGAAAGACCGATAGTCCCTGCCAGCCGATAGGAGACCGTCGTGTAGGCTGGAGCTGCTTC 3’ and RV_60 bp primer: 5’ TGCGGCAGGGCTTGATAATCCTGCCGCATGCCCCTCGACGCATATGAATATCCTCCTTAG 3’ were used. The PCR product from the previous step was then electroporated using Micro Pulser^{TM}, at 2.2 kV into electrocompetent DH5α pKD78 cells containing targeted genomic clone, to induce λ Red genes mediated homologous recombination. Kanamycin resistant recombinants were selected and deletion constructs were confirmed by PCR using the forward or reverse primers from the initial PCR cloning in combination with one of the two primers located inside the kanamycin resistance gene, PSk1 (5’ CAGTCATAGCCGAAT AGCCT 3’) and PSkt′ (5’ GGATTCATCGACTGTGGCCG 3’) (Datsenko & Wanner, 2000). The resulting deletion constructs for each gene were transferred from DH5α pKD78 to the wild type *B. japonicum* USDA110 by tri-parental mating with the helper plasmid pRK2073. Transconjugants generated from double-crossover homologous recombination were selected with the help of *sacB* marker and

resistance to kanamycin and chloramphenicol. The mutant strains were confirmed by PCR using the same primer combination used for deletion constructs confirmation.

Comparison of growth rate. *B. japonicum* USDA 110 (wild type), *blr2694* mutant and *blr75933* mutant strains were grown in 10 ml of AG medium (pH 6.8) at 30°C with shaking at 200 rpm for 2 days with proper antibiotics until reach mid-log phase and then subcultured in 50 ml of AG media with proper antibiotics. Three replicates for each strain were incubated at 30°C with shaking at 200 rpm for 5 days until the cultures reach the stationary phase. OD600 measurements and CFU counting was performed in every 12 h interval during the experimental period. This experiment was repeated three times.

Survivability test under acid shock. The wild type, *blr2694* mutant and *blr7593* mutant strains were grown in 10 ml of AG medium (pH 6.8) at 30°C with shaking at 200 rpm with proper antibiotics until reach mid-log phase and then subcultured in 200 ml of AG media with proper antibiotics. Three replicates of each strain were incubated at 30°C with shaking at 200 rpm until the cultures reach OD600 0.8-1.0. Cultures at OD600 of 0.8 were divided into two flasks 100 ml of culture in each. Then 500 µl of 2N HCl was added to 100 ml of culture and incubated at 30°C with shaking at 200 rpm for 4 h. Five hundred microliters of sterilized distilled water were added into the other 100 ml of culture strain was maintained at 30°C with shaking at 200 rpm for 4 h as the control group. OD600nm and CFU/ ml was monitored at 0 time point and after 4 h.

RESULTS

Acid shock induced cell death and survivability. *B. japonicum* USDA110 that grown until mid-log phase at 30°C in AG medium (pH 6.8) was exposed to different acidic levels instantaneously to study how it behaves in response to acid shock conditions. In this study cells were subjected to different acidic levels by exposing cells in 100 ml of AG media to 500 µl of 1N,

2N, 4N, 6N, 8N, 10N and 12.1N HCl and 500 µl of sterilized distilled was added into the control group. pH change of each treatment condition was measured over time (Fig.3-1). Growth of the bacterium was monitored in different time intervals after exposing to the selected acidity. According to the results (Fig.3-2) adding 500 µl of 2N HCl in to 100 ml of the *B. japonicum* USDA110 culture dropped the culture pH up to 3.5 - 4.0 pH or approximately 3.7 pH. Considering the number of cells survived, exposing the cells to 500 µl of 2N HCl in to 100 ml or pH 3.7 for 4 h was finalized as the stressful condition for *B. japonicum* USDA110 in this study. Adding 500 µl of 3N HCl in to 100 ml was done to confirm the above results and that showed the complete cell death of the bacteria after 4 h. Even though the OD_{600} did not show much difference than that of the 2N HCl (Fig. 3-3, Fig. 3-4). Therefore, exposing the cells to 500 µl of 2N HCl in to 100 ml or pH 3.7 for 4 h was used to study the gene expression of *B. japonicum* USDA110.

Whole genome transcriptional analysis of *B. japonicum* USDA110 cells exposed to heat stress. Microarray analysis was done to study the gene expression of *B. japonicum* USDA110 in response to acid shock for 4 h after adding 500 µl of 2N HCl into 100 ml of culture medium. To identify the genes that were specifically regulated in response to acid shock, gene expression of the cells (100 ml of AG media) exposed to 500 µl of 2N HCl for 4 h was compared to that of the cells (100 ml of AG media) exposed to 500 µl of sterilized distilled water for 4 h after reaching mid-log phase. Microarray analyses indicated that of the 8, 480 *B. japonicum* USDA110 ORFs analyzed, 1136 were differentially expressed more than 2.0 cut-off ($p < 0.05$). Among them, 582 genes were uniquely up-regulated while 619 genes were significantly down-regulated in total (Table 3-4). Genes that were significantly up-regulated and down-regulated in response to acid shock, can be categorized into 15 functional groups (Fig.3-6). The highest number of stimulated and suppressed genes encoded hypothetical proteins; hence, their specific physiological function

could not be implied. However, a variety of genes encoding proteins involved in cellular processes, energy metabolism, regulatory functions, translation, and transport were significantly regulated in response to acid shock. Among those groups, 66 genes involved in translation and 35 regulatory functions related genes were up-regulated while more cellular processes associated (81 genes), and transport binding protein coding genes (65 genes) were down-regulated. There was no difference between the number of genes that were up and down regulated in relation to amino acid biosynthesis. Few genes, 2 and 5 that are responsible for DNA replication, recombination, and repair were up and down regulated, respectively. Purines, pyrimidines, nucleosides, and nucleotides synthesis related genes were very lowly expressed comparatively other functional groups where only 1 gene was up regulated while 4 genes were suppressed. Further, there was not much difference between the up and down regulated number of genes related to fatty acid, phospholipid, and sterol metabolism where 7 genes were up-regulated while 12 genes were down-regulated. Additionally, 10 genes and 2 genes that are involved in transcription were up and down regulated (Table 3-4.).

Among the up-regulated genes in response to acid shock, two genes coding for multidrug resistance efflux pumps *blr7593* and *bll6622* were significantly expressed in 37.87 and 22.11-fold, respectively (Table 3-5). Further, ABC transporter phosphate-binding protein (*blr1091*), serine protease DO-like protease (*blr2591*), small heat shock protein (*bll0729*) and transcriptional regulatory protein (*bll5689*) which were significantly up regulated in 31.37, 20.78, 13.44 and 12.07-fold, respectively were among the top 20 up regulated genes. Additionally, there were many other genes which are significantly expressed more than 2-folds in response to acid shock such as cold shock protein *bsl4595*, serine protease DO-like precursor *blr3130*, small heat shock proteins *blr7740* and *blr5221* which were also induced under heat stress in this study, sigma32-like

transcription factor *blr7337*, glutathione S-transferase like protein *bll4398* and 50S ribosomal protein L21 *blr0420*. Interestingly, *blr2694* VirG like two component response regulator coding gene which relates to nitrogen fixation was induced in 8.39 folds.

Among the down regulated genes in response to acid shock number of genes that are responsible for chemotaxis or cellular movement were suppressed in more than 2.0 folds such as flagellar hook protein *bsl6587*, hook formation protein *bll6853*, pilus assembly protein pilin subunit *bsl1442*, probable PilA2 pilus assembly protein *bsl3118*, components of type IV pilus pilin subunit *bsl7141*, hook associated protein I homolog *bll6857*, flagellin coding genes *bl6866* and *bll6865*, flagellar basal-body rod protein *bll5813*, flagellar hook-basal body complex protein *bll6874* and flagellar basal-body rod protein *bll6876*. Most of these genes are related to flagellar and pilus protein coding operons. Additionally, genes such as putative cell division inhibitor proteins *blr4211*, *bll6598*, chromosome segregation protein *bll2496* related to cell division were also suppressed (Table 3-6).

According to the mutagenesis studies *blr2694* mutant and *blr7593* mutant showed the same growth rate as the wild type indicating that the two genes are not important for growth under regular conditions. However, survivability% of the mutant *blr7593* was significantly different than that of the wild type showing that *blr7593* is vital for the survival of *B. japonicum* USDA110 under acid shock. In contrast, *blr2694* gene was proved to be not necessarily important for survival under acid shock.

DISCUSSION

This study was conducted to investigate the acid shock responses of *B. japonicum* USDA110, the N fixing microsymbiont of soybean plant. Soil acidity is one of the major constraints that affects nodulation, N fixation of rhizobia and their persistence and multiplication

in soil (Martinez-Romero et al., 1991). Acidic soils are always associated with low levels of essential nutrients such as manganese (Mn), but also nutrient deficiency by low levels of phosphorus (P), potassium (K), calcium (Ca), molybdenum (Mo), and magnesium (Mg) and high levels of toxic elements such as aluminium (Al) (Martinez-Romero et al., 1991). Researchers have attempted to isolate both plants and rhizobia that can tolerate soil acidity. In terms of rhizobia, fast growing rhizobia is considered as less tolerant to acidity than slow growing bradyrhizobia. *B. japonicum* is one of the most effective rhizobium species (Asanuma & Ayanaba, 1990). However, *Rhizobium loti* and *Rhizobium tropici* (Martinez-Romero et al., 1991) have also been proved highly acid tolerant, with growth at pH 4.0 (Cooper, 1982; Cunningham & Munns, 1984; Karanja & Wood, 1988). Strains of *Rhizobium meliloti* are also susceptible to soil acidity and their numbers are affected soil (Brockwell et al., 1991).

pH is a critical factor that governs the persistence of free-living rhizobia in soil. In this study, a series of growth curve experiments was conducted to observe the behavior of *B. japonicum* USDA110 in response to different pH shock conditions, to find the stressful condition for the bacterium that should be used in the gene expression study. Results of the growth curve analysis showed that the bacterium is unable to survive upon exposure to lower pH levels such as pH 2.5. Number of cells survived began to decline rapidly after few hours of exposure to pH 2.5 or 500 µl of 3N HCl and cells were completely dead after 4 h. Cells were able to survive in a decreased rate after exposing to 500 µl of 3N HCl for 4 h. According to the survivability data under different acidic levels at different time intervals indicated that the cells are completely dead after exposing to 500 µl of 3N HCl for 4 h leaving, 500 µl of 2N HCl for 4 h as the stressful condition for *B. japonicum* USDA110 under which it can thrive at a reduced rate. That experimental condition was

chosen to be used in gene expression analysis to explore what genes and mechanisms made it to subsist the stress condition.

During the microarray studies *blr7593* and *bll6622* multidrug resistance efflux pumps coding genes were significantly upregulated where blr7593 was the highest since it is a prime necessity to pump out the extra protons from the cells to maintain the internal pH stably around neutrality. Number of genes and operons responsible for flagellar and pilus formation were significantly repressed to save cellular energy for production of chaperones and other proteins requires to overcome the acid shock. Further, it infers that during the acid shock cells tend to be immotile again for the same purpose of energy saving. Moreover, production of proteins related to cell division was also restricted. That could be attributed to the slower growth rate and cell numbers under acid shock.

During the process of evolution, microorganisms have developed sophisticated physiological and molecular level adaptations to recover from acid stress (Nezhad et al., 2015). Microorganisms have evolved several resistance mechanisms such as pH homeostasis, alteration of cell membranes properties, metabolic regulations, protection, and repair of macromolecules to defend themselves (Guan & Liu, 2020).

pH homeostasis is the regulation of the pH inside and outside the cell. It is important to maintain a relatively stable pH inside the cells for growth and metabolism. It affects the absorption of nutrients, degradation of substrates, and synthesis of macromolecules such as nucleic acids and proteins. Maintaining of the pH homeostasis is achieved by the interactions of multiple transport systems. Proton pumps expel protons out from cells generating a membrane potential and a pH gradient. These two processes are interchanged and regulated by secondary transporters. Microbes deployed different strategies to maintain pH homeostasis. For example, maintaining relatively

stable and neutral intracellular pH than the external pH through generation of proton gradients is found in some bacteria and yeast cells (Siegumfeldt et al., 2000). Most acid-tolerant microorganisms prefer to maintain a constant pH gradient since maintaining a neutral internal pH is highly energetically costly which restraints growth and metabolic activities of bacteria. Continuous acidification of the environment gradually affects the stability of internal neutral pH and destroys the pH homeostasis at a certain level where the internal pH begins to decline rapidly. Consequently, damaged protein and DNA lead the cells to collapse ultimately. Therefore, it's important to maintain pH homeostasis stable for survival of bacteria under acid shock (Wu et al., 2012). Acid tolerant microorganisms possess the ability to control permeability of plasma membrane to protons to reduce the proton entry into the cells. To achieve this, microbes adopt several structural modifications such as tough monolayer, the isoprenoid core, and unique lipid composition such as tetraether lipids (Macalady & Banfield, 2003). Modulating the size of membrane channel (Amaro et al., 1991), generating chemiosmotic gradient (Baker-Austin & Dopson, 2007), and exchanging H^+ and K^+ via cation ATPases (Macpherson et al., 2005) are some other pathways of maintaining of pH homeostasis. Additionally, pumping out excess protons from the cell through proton motive force dependent proton pumps and consumption of excessive cytoplasmic protons are other important systems, found in bacteria in maintaining pH homeostasis (Jain et al., 2013; Mols & Abee, 2011). Moreover, amino acid-dependent acid tolerance systems are functioning under acidic conditions to raise internal pH in some microorganisms (Senouci-Rezkallah et al., 2011).

Apart from the monitoring of proton exchange through cell membrane, modification of the integrity, fluidity, and lipid composition of cell membranes also protect bacteria from deleterious effects of acidity (Yan et al., 2016). Cytoplasmic membrane ensures a stable intercellular

environment independent from the external environmental fluctuations. Low pH could damage the integrity and fluidity of cell membrane leading to morphological changes in cells. Structure, composition, and distribution of fatty acids in the cytoplasmic membrane determine and maintain the membrane fluidity which confer acid tolerance (Denich et al., 2003; Yang et al., 2014).

Microorganisms can prioritize the metabolic pathways during the cell adaptation for acidic conditions to protect itself. They strengthen the glycolytic pathway by increasing the glycolytic rate to upgrade precursors, co-factors and redox factors required for survival, growth, and metabolism under acidic stress. Further, enzyme concentration and activity are also increased to resume normal cellular activities (Even et al., 2003). Proteins and DNA can be irreversibly damaged and loss their specific functions due to acidity. Hence, protection and repair of proteins and DNA is prime important when cells are under acidification. Denaturation of proteins is a common consequence of cell exposure towards most of the abiotic stresses. The enzymes, transporters and transmembrane antiporters reside in the periplasm of Gram-negative bacteria directly subjected to severe acid stress because of lacking the protection of inner membrane. Acidity causes denaturation and aggregation of these proteins. HdeA and HdeB are two periplasmic molecular chaperones that involve in protecting proteins from acid induced denaturation. HdeA chaperone get activated under acidic pH and prevents the aggregation of protein by binding to them. It is also involved in protein resolubilization and renaturation. DegP and SurA are two other chaperones that assist HdeA to restore the activity of proteins by assisting refolding during renaturation. HdeB also has the same function as HdeA under acid stress responses of bacteria. (Hong et al., 2012). Lo 18 is a membrane-bound heat shock protein identified in *Oenococcus oeni* which functions as a molecular chaperone to stabilize membrane and envelop proteins under acidic conditions (Weidmann et al., 2017).

Biofilm formation is another fascinating mechanism utilized by some bacteria to protect themselves against acid shock. This is a social behavior that the cells are wrapped and protected in the innermost part of the biofilm which depends on the cell density (Li et al., 2001).

Table 3- 1. List of bacterial strains and plasmids.

Strain or Plasmid	Genotype or phenotype	Reference
***E. coli* strain**		
DH5α	supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 F' (traD36, proAB+ lacIq, Δ(lacZ)M15) endA1 recA1 hsdR17 mcrA supE44λ gyrA96 relA1	Bethesda Research Laboratories
***Bradyrhizobium* sp.**		
B. japonicum USDA110	Cm^R Wild type	USDA
Δ*blr2694* mutant	Cm^R Km^R *blr2694* :: Km	This work
Δ*blr7593* mutant	Cm^R Km^R *blr7593* :: Km	This work
Plasmids		
pKD4	Km^R expression vector	(Datsenko and Wanner 2000)
pKD78	Cm^R Lambda Red recombinase expression plasmid	(Datsenko and Wanner 2000)
pRK2013	Km^R cloning vector	(Ditta et al.1980)
pJQ200SK	Gm^R *sacB* suicide vector	(Quandt and Hynes 1993)
pJQ200SK- *blr2694*	Gm^R suicide vector containing *blr2694* gene	This work
pJQ200SK- *blr7593*	Gm^R suicide vector containing *blr7593* gene	This work
pKD78-pJQ200SK-*blr2694-λ*	Gm^R Cm^R suicide vector containing *blr2694* gene with Lambda Red recombinase expression plasmid	This work
pKD78-pJQ200SK-*blr7593-λ*	Gm^R Cm^R suicide vector containing *blr7593* gene with Lambda Red recombinase expression plasmid	This work
pKD78-pJQ200SK-*blr2694*-Km	Gm^R Km^R suicide vector containing *blr2694* gene substituted with kanamycin cassette	This work

pKD78-pJQ200SK-*blr7593*-Km	Gm^R Km^R suicide vector containing *blr7593* gene substituted with kanamycin cassette	This work

*Cm, Km and Gm refer to chloramphenicol, kanamycin, and gentamycin, respectively.

Table 3-2. List of candidate genes selected for qPCR from *B. japonicum* USDA110 microarray study.

Gene name	Function
blr7593	multidrug resistance efflux pump
blr2591 (dop)	serine protease DO-like protease
bsl0708 (rpmI)	50S ribosomal protein L35
blr7337 (rpoH2)	sigma32-like transcription factor
blr5220 (hspE)	small heat shock protein
bll5416 (nusG)	transcription antitermination protein
bll4012	organic hydroperoxide resistance protein
bll4517	probable DNA polymerase III delta prime subunit
bll0322 (otsA)	probable trehalose-6-phosphate synthase
blr2272	putative porin precursor
bsl4127	cold shock protein
blr1171 (coxA)	cytochrome C oxidase subunit I
bll6866 (fla)	flagellin
bll4906 (nuoL)	NADH ubiquinone oxidoreductase chain L
bll0829 (dnaN)	DNA polymerase III beta chain

Table 3-3. List of primers used for qPCR genes.

Gene name	Forward primer	Reverse primer
blr7593	TCGAACTGCAACAGCCGATCAT	TTCCTCCTGGGCGAATTTCAGA
blr2591 (dop)	CTCCGGCTTCTTCATCTCAG	GCTTCTGGTCGGAGAACTTG
bsl0708 (rpmI)	CCCAAGCTGAAGACCAAATC	CGTCGGTCTTGAACAGCAC
blr7337 (rpoH2)	AACTCGTCACCAGCCATCTC	CCAGGAACGCAGGATGTACT
blr5220 (hspE)	AGACAACGGCGACACTTACC	ACAGGTAGTCGCGTTCATCC
bll5416 (nusG)	GGTACATCGTCCACGCCTAT	GATCAGATGAAACGCCTCGT
bll4012	TGCCAAGCTTCTCTTTACCG	TGCCAAGCTTCTCTTTACCG
bll4517	GCTAACGCGCTTCTGAAGAT	ACTGATCACCTCGTCCGTTC
bll0322 (otsA)	GGCAATCTTCAGAACGAGGT	TACTCCTTTGCGACGAGGTT
blr2272	GCAGAAGCTTTTCGATGACC	CTCACCGGCCAGTTGTAGAT
bsl4127	AGGGCTATGGGTTCATCAAA	GTTCTCCGCGGATGATTTT
blr1171 (coxA)	AACATCTCGTTCTGGCTGCT	CGTGGTGATGAAGTTGATGG

bll6866 (fla)	GGTATCGATCGCTCCAAGATCCA	TCAGCCAGTTGATGCCGTTGAT
bll4906 (nuoL)	AACCTCGTGCAGCTGTTCTT	AGCATGAAGATCGCGAAGAT
bll0829 (dnaN)	CGTCGTCGACAAGAAGGATT	GCAGATAGCGGGAGTTGAAG

Table 3-4. Functional roles of significant genes responding to acid shock.

Functional Groups	Up	Down
Amino Acid Biosynthesis	8	8
Biosynthesis of cofactors, prosthetic groups, and carriers	13	7
Cell envelope	4	17
Cellular processes	25	81
Central intermediary metabolism	15	10
DNA replication, recombination, and repair	2	5
Energy metabolism	24	25
Fatty acid, phospholipid, and sterol metabolism	7	12
Purines, pyrimidines, nucleosides, and nucleotides	1	4
Regulatory functions	35	24
Transcription	10	2
Translation	66	5
Transport and binding proteins	31	65
Other categories	64	58
Hypothetical	277	296
Total	582	619

Table 3-5. Significantly up-regulated genes in response to acid shock with 2NHCl for 4 h.

Gene Name	**Fold Change**	**Description (Level III)**
blr7593	37.87	multidrug resistance efflux pump
bll5859	35.19	DUF1127 domain-containing protein
blr1091	31.37	ABC transporter phosphate-binding protein
bsr4236	30.91	hypothetical protein bsr4236
bsl4014	22.21	hypothetical protein bsl4014
bll6622	22.11	multidrug resistance efflux pump
blr2591	20.78	serine protease DO-like protease
blr4827	19.46	hypothetical protein blr4827
bll1466	15.14	DUF883 domain-containing protein
bsl4437	13.83	hypothetical protein bsl4437
bsl7903	13.80	DUF1127 domain-containing protein
blr7528	13.76	DUF2076 domain-containing protein
bsl2206	13.50	hypothetical protein bsl2206
bll0729	13.44	small heat shock protein
bll5689	12.07	transcriptional regulatory protein

Table 3-6. Significantly down-regulated genes in response to acid shock with 2NHCl for 4 h.

Gene Name	Fold Change	Description
bll0333	-21.65	Probable alcohol dehydrogenase precursor
blr2272	-17.48	Putative porin precursor
blr3208	-16.67	ABC transporter sugar binding protein
bsl6587	-14.41	Components of type IV pilus pilin subunit
bll6858	-14.16	Flagellar hook protein
bll4784	-13.43	Aldehyde dehydrogenase
bsr1505	-12.30	Hypothetical protein bsr1505
blr7827	-11.93	ABC transporter substrate binding protein
bsl4127	-11.28	Cold shock protein
blr3200	-10.73	ABC transporter sugar binding protein
bll5076	-10.58	Hypothetical protein bll5076
bll6853	-10.28	Hook formation protein
blr4701	-10.25	Putative outer-membrane immunogenic protein precursor
bll0912	-10.04	Hypothetical protein bll0912
blr2269	-9.91	ABC transporter sugar binding protein

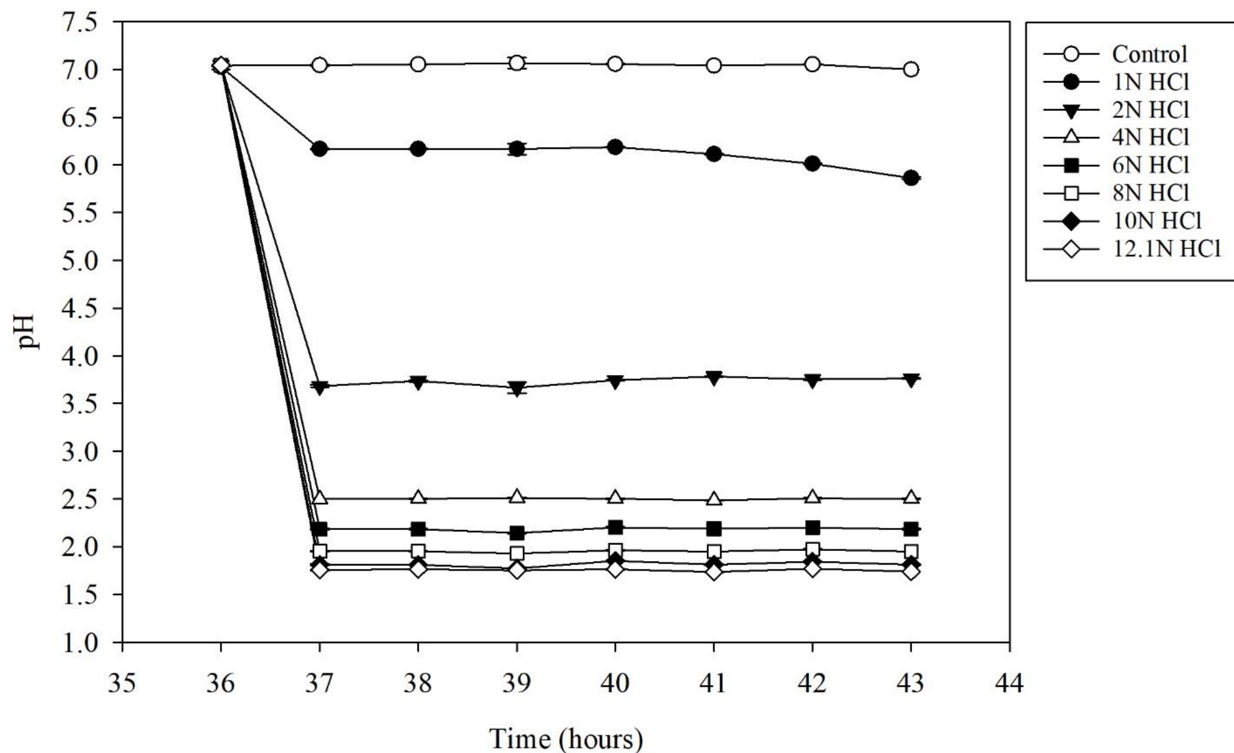

Figure 3-1. Changes of pH in 100 ml AG culture media (pH 6.8) upon addition of 500 µl of HCl with different concentrations over time. Each time point represents the average pH of three replicates. Error bars represent standard deviation of the means Symbols: ○, Control (500 µl of sterilized ddH_2O); ●, 1N HCl; ▼, 2N HCl; ∆, 4N HCl; ■, 6N HCl; □, 8N HCl; ◆ , 10N HCl; ◇ , 12.1N HCl.

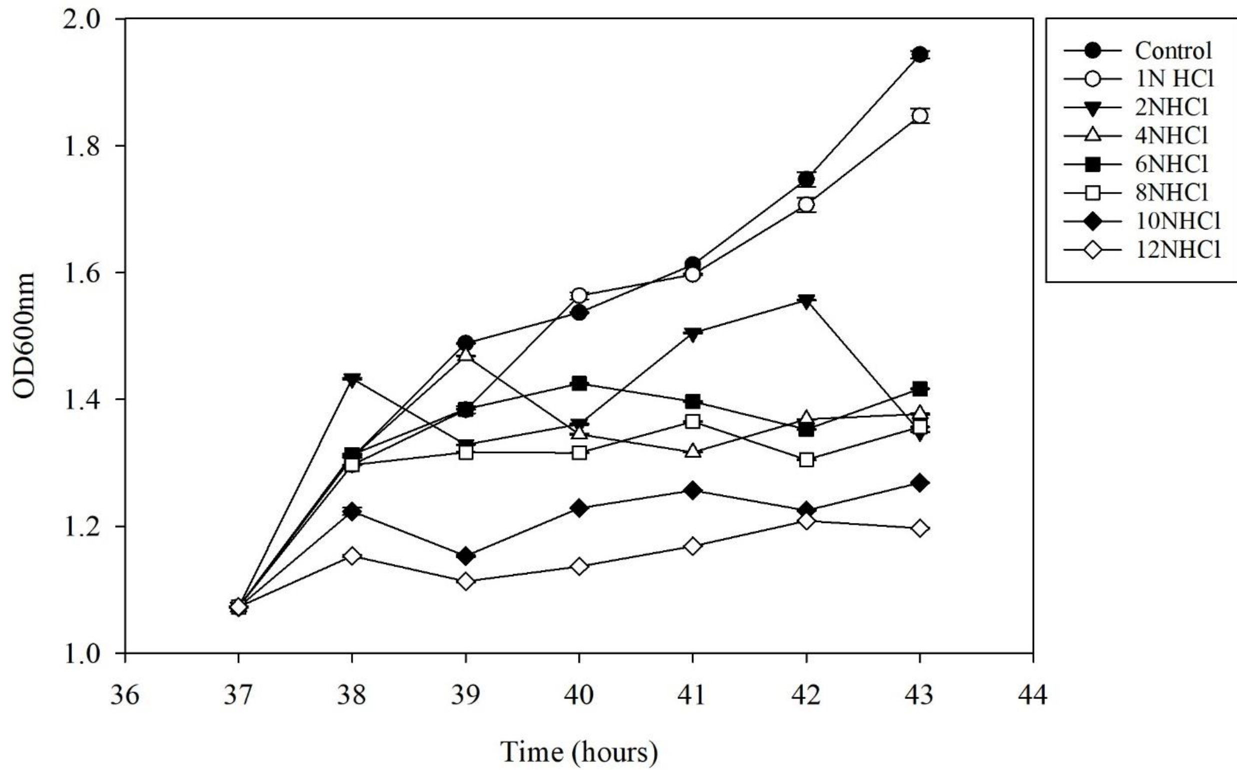

Figure 3-2. Growth of *B. japonicum* USDA110 in 100 ml of AG media (pH 6.8) upon exposure to acid shock by addition of 500 µl of HCl with different concentrations over time. Each time point represents the average OD of three replicates. Error bars represent standard deviation of the means Symbols: ○, Control (500 µl of sterilized distilled water); ●, 1N HCl; ▼, 2N HCl; Δ, 4N HCl; ■, 6N HCl; □, 8N HCl; ◆, 10N HCl; ◇,12.1N HCl.

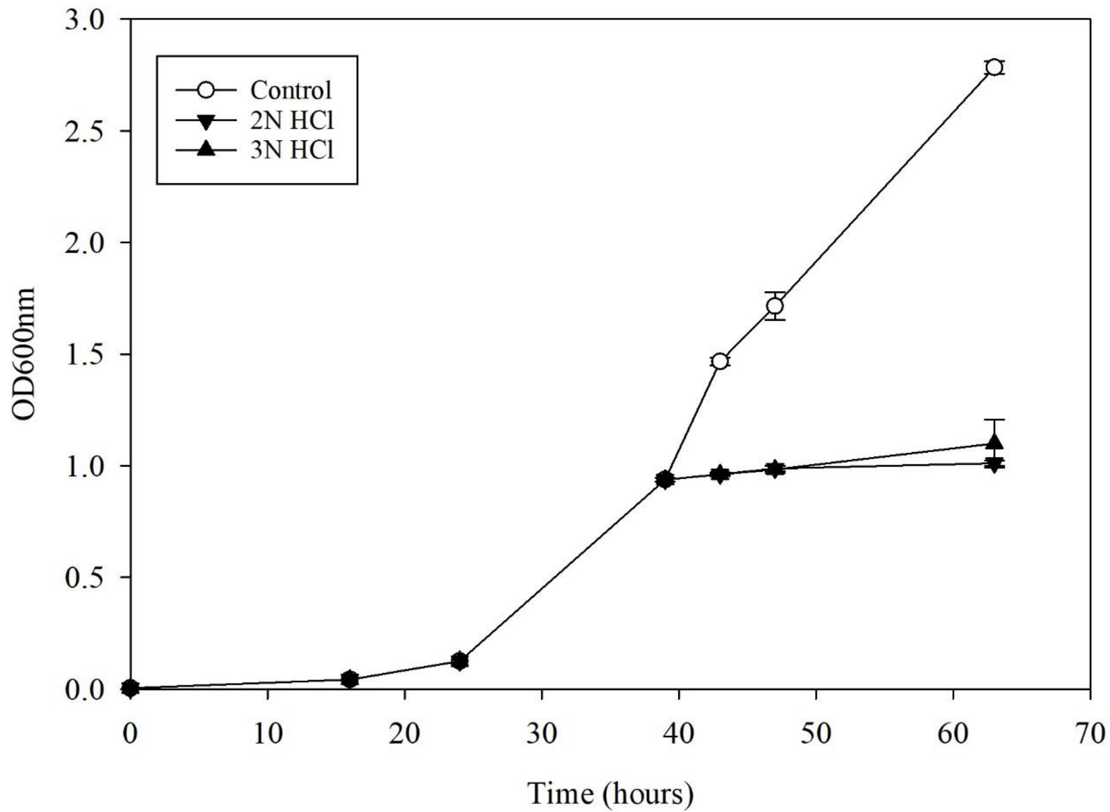

Figure 3-3. Growth of *B. japonicum* USDA110 in 100 ml of AG media (pH 6.8) upon exposure to acid shock by addition of 500 µl of 2N HCl and 3N HCl over time. Each time point represents the average OD of three replicates. Error bars represent standard deviation of the means Symbols: ○, Control (500 µl of sterilized distilled water); ▼, 2N HCl; ▲, 3N HCl.

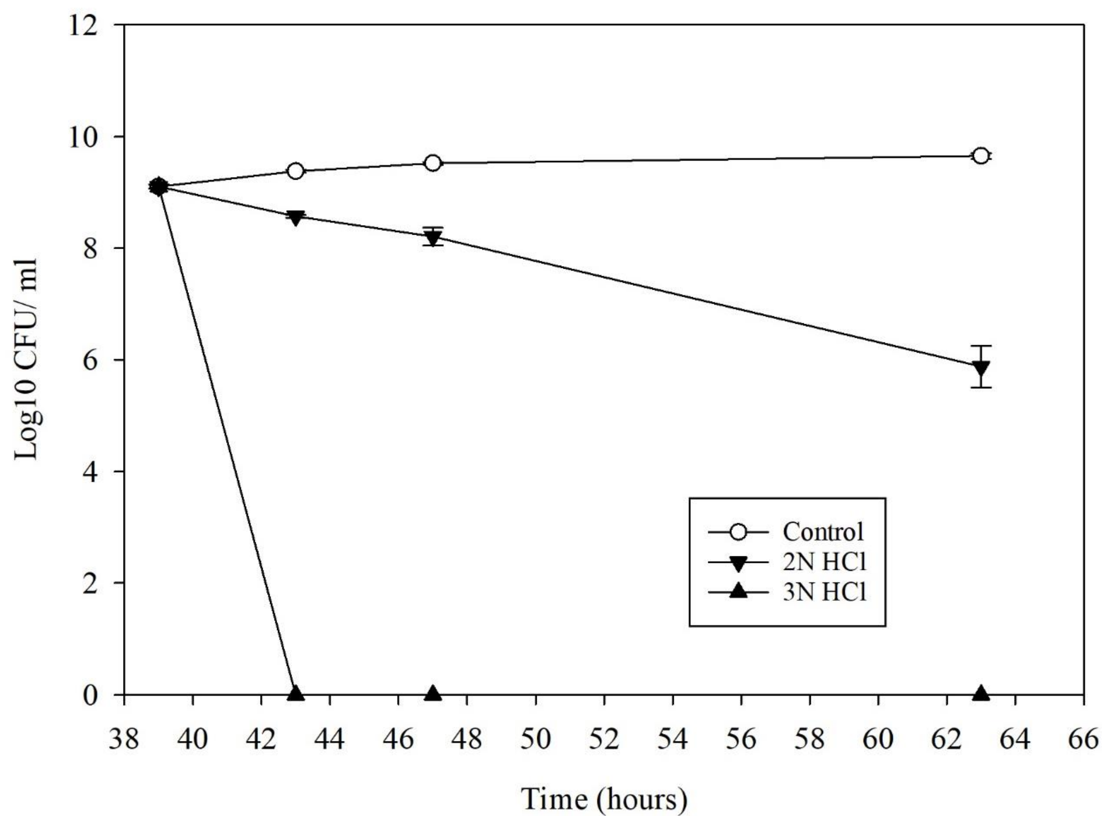

Figure 3-4. Growth of *B. japonicum* USDA110 in 100 ml of AG media (pH 6.8) upon exposure to acid shock by addition of 500 µl of 2N HCl and 3N HCl over time. Each time point represents the average of log10 transformed CFU/ ml of three replicates. Error bars represent standard deviation of the means. Symbols: ○, Control (500 µl of sterilized distilled water); ▼, 2N HCl; ▲, 3N HCl.

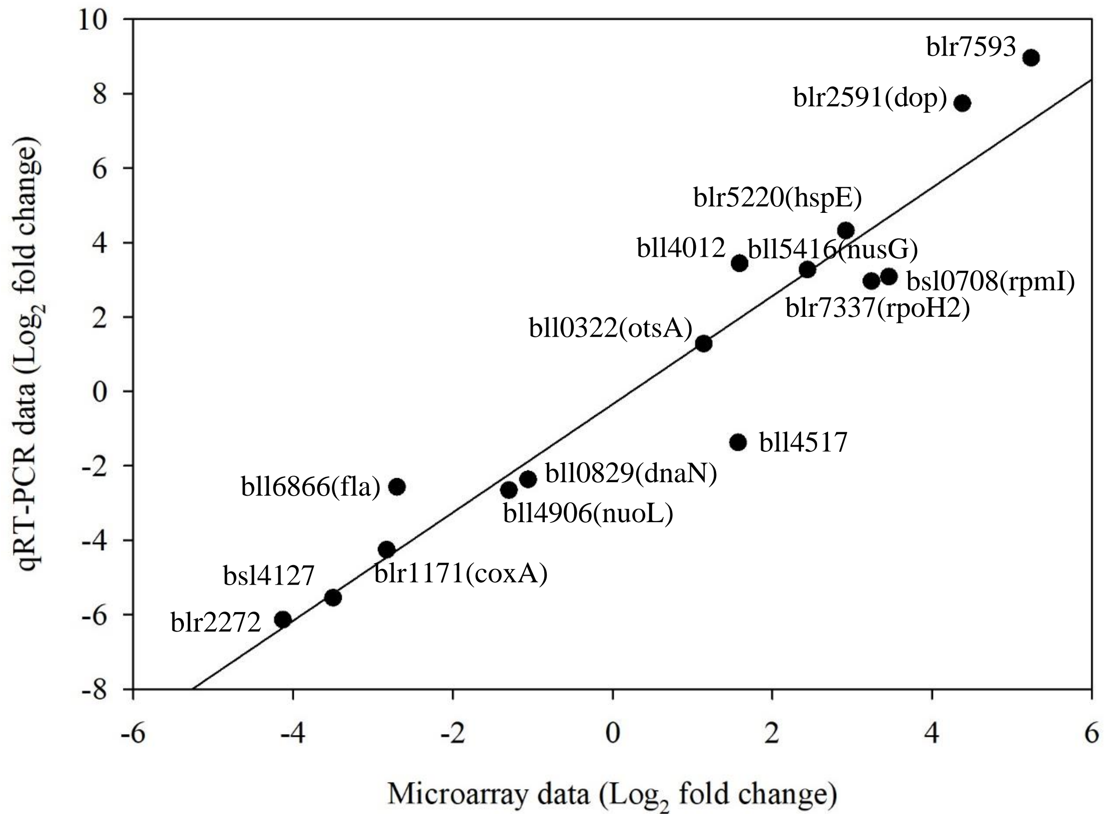

Figure 3-5. Correlation between microarray and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) data of *B. japonicum* USDA110 for 15 genes. These genes were chosen based on fold induction and functional categories. Fold change values were log2 transformed.

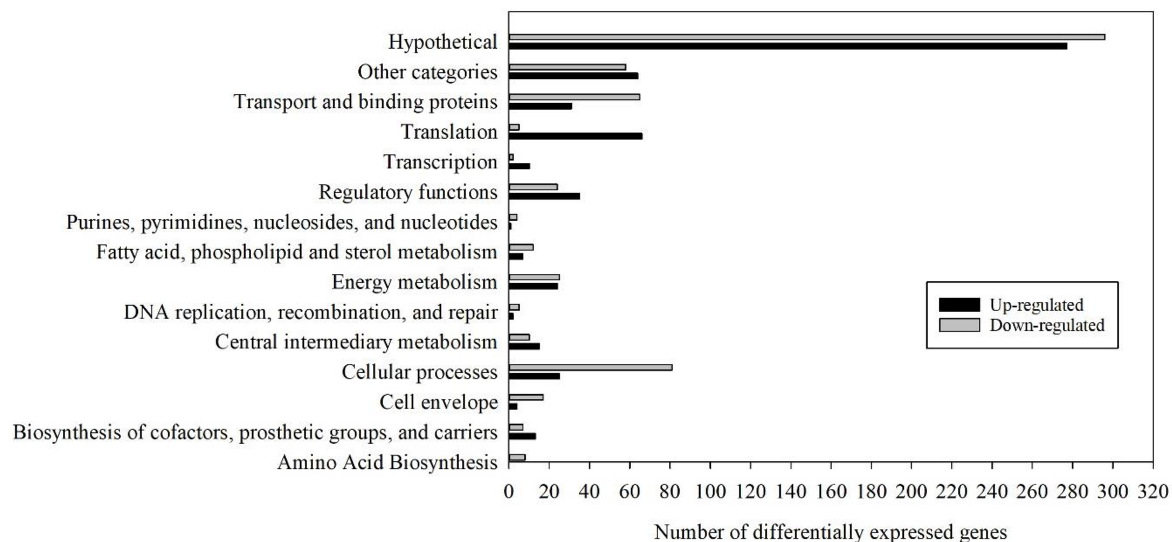

Figure 3-6. Functional categories of statistically significant, differentially expressed genes under acid shock at 500 µl of 2N HCl in 100 ml of AG media for 4h. Black bars, up-regulated genes; gray bars, down-regulated genes.

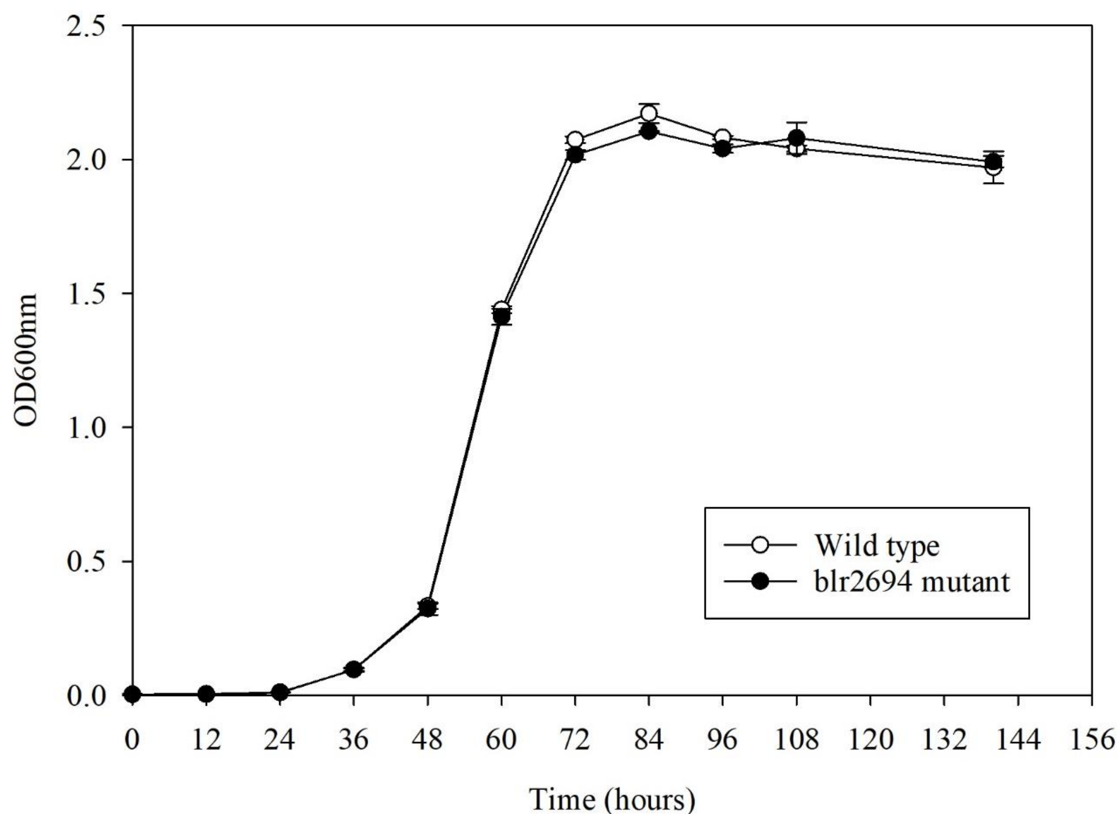

Figure 3-7. Growth comparison of the *blr2694* mutant and the wild type *B. japonicum* USDA110 in AG medium (pH 6.8) at 30°C. Each time point represents the average OD of three biological replicates. Error bars indicate the standard deviation of means. Symbols: ○, wild type; ●, *blr2694* mutant.

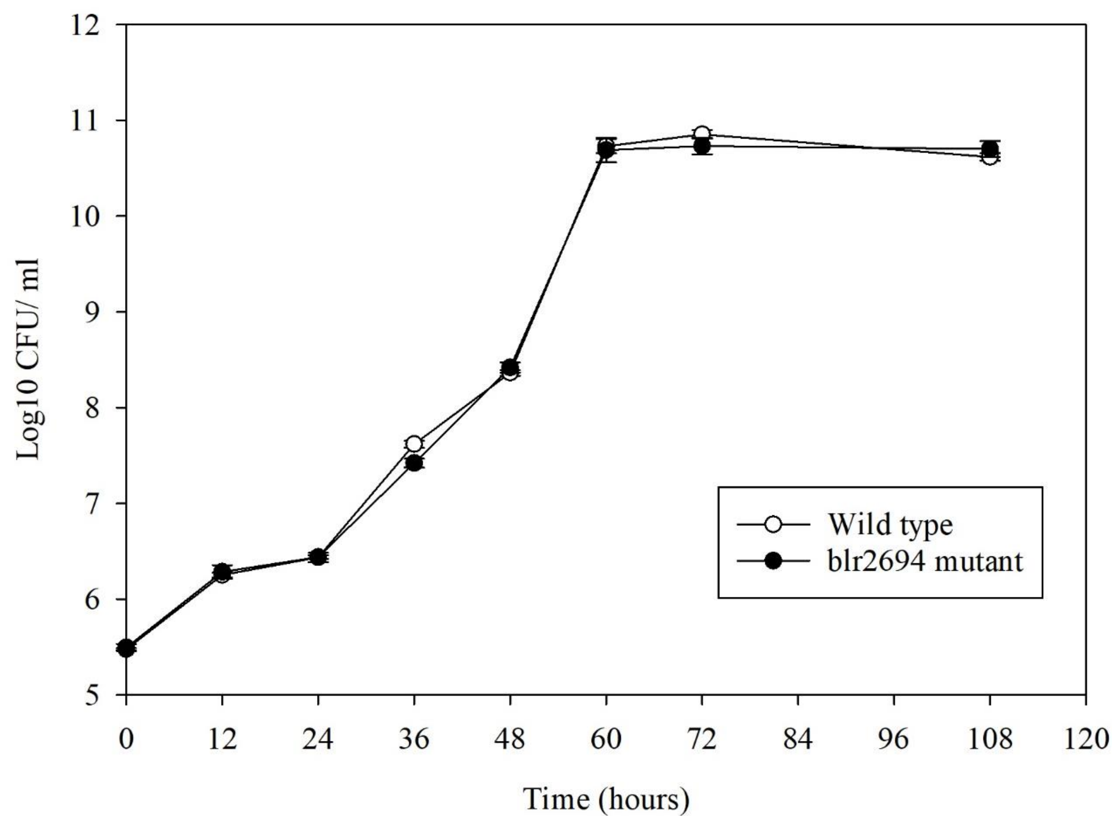

Figure 3-8. Growth comparison of the *blr2694* mutant and the wild type *B. japonicum* USDA110 in AG medium (pH 6.8) at 30℃. Each time point represents the average log10 transformed CFU/ ml of three biological replicates. Error bars indicate the standard deviation of means. Symbols: ○, wild type; ●, *blr2694* mutant.

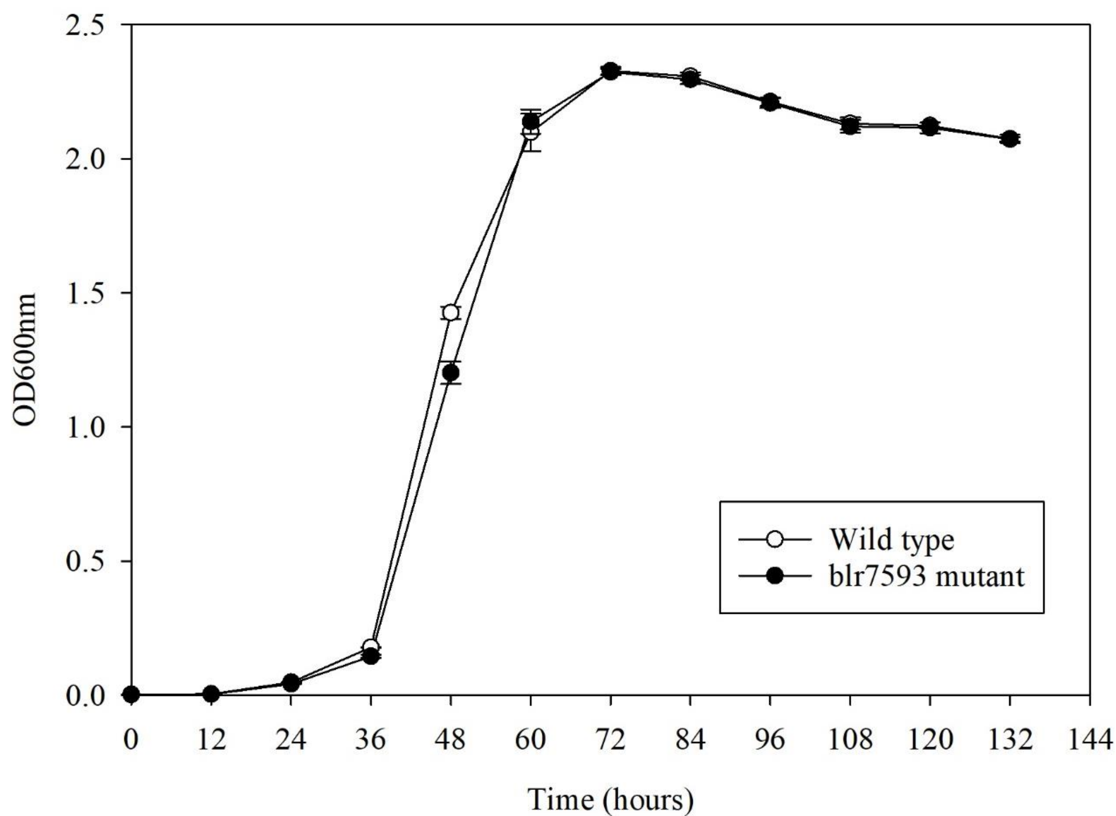

Figure 3-9. Growth comparison of the *blr7593* mutant and the wild type *B. japonicum* USDA110 in AG medium (pH 6.8) at 30°C. Each time point represents the average OD of three biological replicates. Error bars indicate the standard deviation of means. Symbols: ○, wild type; ●, *blr7593* mutant.

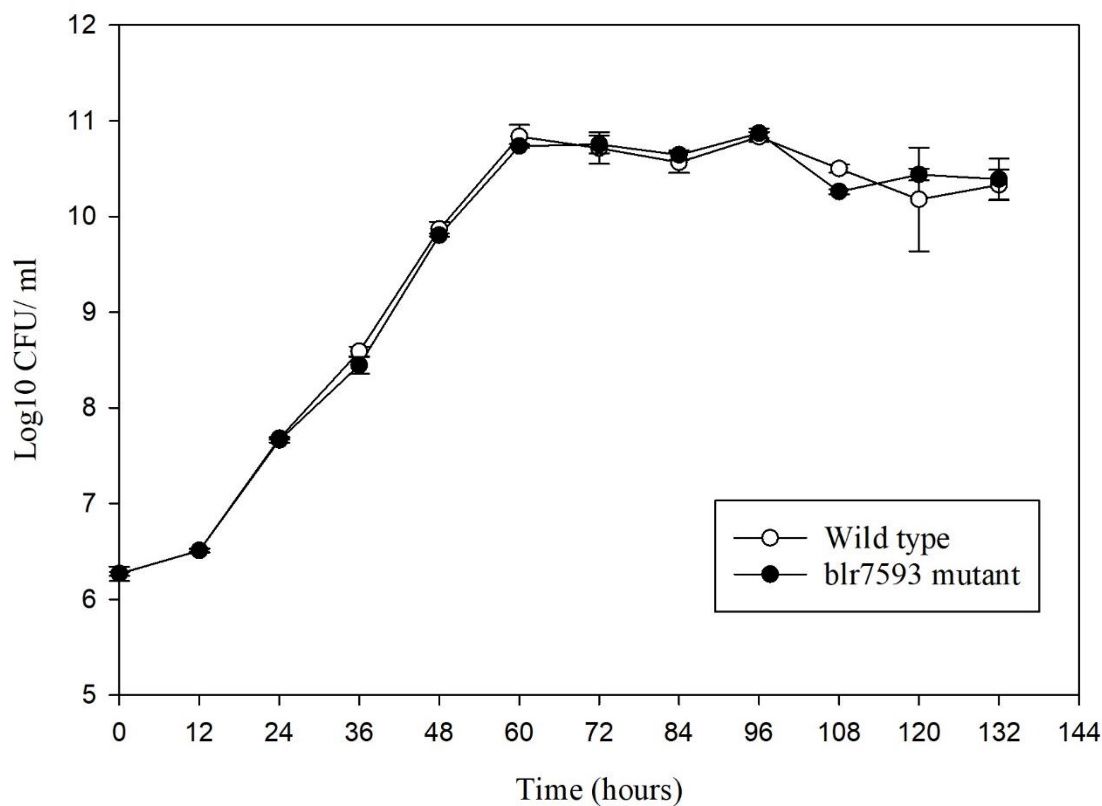

Figure 3-10. Growth comparison of the *blr7593* mutant and the wild type *B. japonicum* USDA110 in AG medium (pH 6.8) at 30°C. Each time point represents the average log10 transformed CFU/ ml of three biological replicates. Error bars indicate the standard deviation of means. Symbols: ○, wild type; ●, *blr7593* mutant.

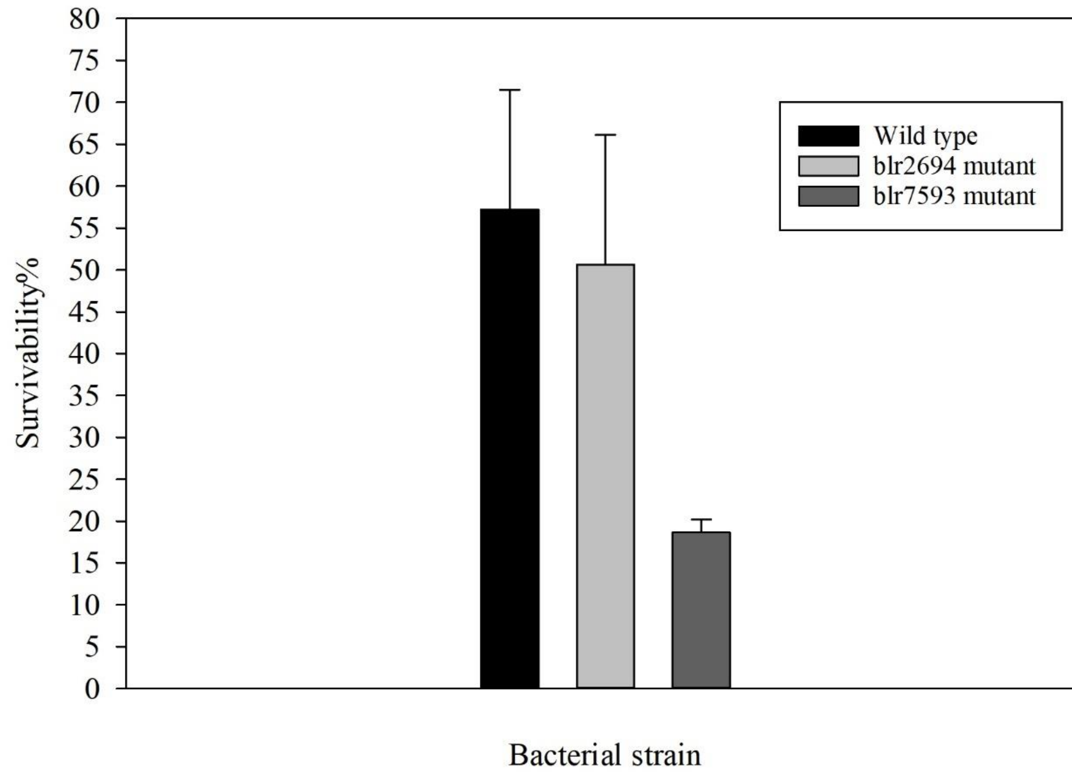

Figure 3-11. Survivability comparison of wild type, *blr2694* & *blr7593* mutants in response to acid shock by exposing the cultures to 500 µl of 2N HCl in 100 ml of AG media for 4 h.

REFERENCES

Aarons, S. R. 1991. Response of *Rhizobium ieguminosarum bv phaseoli* to acidity. *Plant Soil*, **134**, 145–151.

Amaro, A. ., Chamorro, D., Seeger, M., Arredondo, R., Peirano, I., & Jerez, C. . 1991. Effect of External pH Perturbations on In Vivo Protein Synthesis by the Acidophilic Bacterium *Thiobacillus ferroxidans*. *J. Bacteriol.,* **173**(2), 910–915.

Anyngo, B., Wilson, J. K., Beynon, L. J., & Giller, E. K. 1995. Diversity of Rhizobia Nodulating *Phaseolus vulgaris* L. in Two Kenyan Soils with Contrasting pHs. *Appl. and Env. Microbiol.* **61(11)**, 4016–4021.

Appunu, C., & Dhar, B. 2006. Symbiotic effectiveness of acid-tolerant *Bradyrhizobium* strains with soybean in low pH soil. *African J. Biotechnol.*, **5**, 842–845.

Asanuma, S., & Ayanaba, A. 1990. Variation in acid-al tolerance of *Bradyrhizobium japonicum* strains from African soils. *Soil Sci. Plant Nutr.*, **36**, 309–317.

Baker-Austin, C., & Dopson, M. 2007. Life in acid: pH homeostasis in acidophiles. *Trends Microbiol.*, **15**, 165–171.

Bertani, G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.*, **62**, 293–300.

Brockwell, J., Pilka, A., & Holliday, R. A. 1991. Soil ph is a major determinant of the numbers of naturally occurring *Rhizobium meliloti* in non-cultivated soils in central new south wales. *Aust. J. Exp. Agric.*, **31**, 211–219.

Bromfield, E. S. P., & Jones, D. G. 1980. Studies on double strain occupancy of nodules and

the competitive ability of *Rhizobium trifolii* on red and white clover grown in soil and agar. *Ann. Appl. Biol.*, **94**, 51–59.

Chang, W.-S., Franck, W. L., Cytryn, E., Jeong, S., Joshi, T., Emerich, D. W., Sadowsky, M. J., Xu, D., & Stacey, G. 2007. An Oligonucleotide Microarray Resource for Transcriptional Profiling of *Bradyrhizobium japonicum*. *Mol. Plant-Microbe Interact. MPMI*, **20**, 1298–1307.

Cooper, J. E. 1982. Acid production, acid tolerance and growth rate of Lotus rhizobia in laboratory media. *Soil Biol. Biochem.*, **14**, 127–131.

Cunningham, S. D., & Munns, D. N. 1984. The Correlation Between Extracellular Polysaccharide Production and Acid Tolerance in *Rhizobium*. *Soil Sci. Soc. Am. J.*, **48**, 1273–1276.

Cytryn, E. J., Sangurdekar, D. P., Streeter, J. G., Franck, W. L., Chang, W. S., Stacey, G., Emerich, D. W., Joshi, T., Xu, D., & Sadowsky, M. J. 2007. Transcriptional and physiological responses of *Bradyrhizobium japonicum* to desiccation-induced stress. *J. Bacteriol.*, **189**, 6751–6762.

Datsenko, K. A., & Wanner, B. L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.*, **97**, 6640–6645.

Denich, T. J., Beaudette, L. A., Lee, H., & Trevors, J. T. 2003. Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. In *Journal of Microbiological Methods*.,52 (2).

Ditta, G., Stanfield, S., Corbin, D., & Helinski, D. 1980. Broad host range DNA cloning

system for Gram-negative bacteria: construction of a gene bank of Rhizobium meliloti. Proc. Natl. Acad. Sci. **77**(1): 7347-7351.

Even, S., Lindley, N. D., & Cocaign-Bousquet, M. 2003. Transcriptional, translational and metabolic regulation of glycolysis in *Lactococcus lactis* subsp. cremoris MG 1363 grown in continuous acidic cultures. *Microbiology*, **149**, 1935–1944.

Ferguson, B. J., Lin, M. H., & Gresshoff, P. M. 2013. Regulation of legume nodulation by acidic growth conditions. *Plant Signal. Behav.*, **8**, 1–5.

Graham, P. H., Draeger, K. J., Ferrey, M. L., Conroy, M. J., Hammer, B. E., Martinez, E., Aarons, S. R., & Quinto, C. 1994. Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. *Can. J. Microbiol.*, **40**, 198–207.

Guan, N., & Liu, L. 2020. MINI-REVIEW Microbial response to acid stress: mechanisms and applications. *Applided Microbiol. Biotechnol.*, **104**, 51–65.

Hong, W., Wu, Y. E., Fu, X., & Chang, Z. 2012. Chaperone-dependent mechanisms for acid resistance in enteric bacteria. *Trends Microbiol.*, **20**, 328–335.

Hungria, Mariangela, & Vargas, M. A. T. 2000. Environmental factors affecting N2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *F. Crop. Res.*, **65**, 151–164.

Jain, P., Jain, V., Singh, A., Chauhan, A., & Sinha, S. 2013. Evaluation on the responses of succinate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase and glucose-6-phosphate dehydrogenase to acid shock generated acid tolerance in *Escherichia coli*. *Adv. Biomed. Res.*, **2**, 75.

Jeon, J.-M., Lee, H.-I., Donati, A. J., So, J.-S., Emerich, D. W., & Chang, W.-S. 2011. Whole-Genome Expression Profiling of *Bradyrhizobium japonicum* in Response to Hydrogen Peroxide. *Mol. Plant-Microbe Interact. MPMI*, **24**, 1472–1481.

Karanja, N. K., & Wood, M. 1988. Selecting *Rhizobium phaseoli* strains for use with beans (*Phaseolus vulgaris* L.) in Kenya: Infectiveness and tolerance of acidity and aluminium. *Plant Soil*, **112**, 7–13.

Kashket, E. R. 1985. Bacteria : a Critical Assessment of Methods. *Annu. Rev. Microbiol.*, **39**, 219–242.

Keyser, H. H., & Munns, D. N. 1979. Tolerance of Rhizobia to Acidity, Aluminum, and Phosphate. *Soil Sci. Soc. Am. J.,* **43, 519-**

Keyser, H. H., Munns, D. N., & Hohenberg, J. S. 1979. Acid Tolerance of Rhizobia in Culture and in Symbiosis with Cowpea. *Soil Sci. Soc. Am. J.*, **43**, 719–722.

Li, Y.-H., Hanna, N. M., Svensäter, G., Ellen, P. R., & Cvitkovitch, G. D. 2001. Cell Density Modulates Acid Adaptation in *Streptococcus mutans*: Implications for Survival in Biofilm. *J. Bacteriol.*, **183**, 6875–6884.

Lie, T. A. 1969. THE EFFECT OF LOW pH ON DIFFERENT PHASES OF NODULE FORMATION IN PEA PLANTS. *Plant Soil*, **31**, 391–406.

Lin, M.-H., Gresshoff, P. M., & Ferguson, B. J. 2020. Systemic Regulation of Soybean Nodulation by Acidic Growth Conditions. *Plant Physio.* **160,** 2028-2039.

Macalady, J., & Banfield, J. F. 2003. Molecular geomicrobiology: Genes and geochemical cycling. *Earth Planet. Sci. Lett.*, **209**, 1–17.

Macpherson, N., Shabala, L., Rooney, H., Jarman, M. G., & Davies, J. M. 2005. Plasma membrane H+ and K+ transporters are involved in the weak-acid preservative response of disprate food spoilage yeasts. *Microbiology*, **151**, 1995–2003.

Martinez-Romero, E., Segovia, L., Mercante, F. M., Franc0, A. A., Graham, P., Marc0, A., & Pardo', A. 1991. Rhizobium tropici, a Novel Species Nodulating Phaseolus vulgaris L. Beans and Leucaena sp. Trees. *Inter. J. Syst. Bacteriol.* **41(3)**, 417-426.

Mols, M., & Abee, T. 2011. Bacillus cereus responses to acid stress. *Environ. Microbiol.*, **13**, 2835–2843.

Nezhad, H. M., Hussain, M. A., & Britz, M. L. 2015. Stress Responses in Probiotic Lactobacillus casei. *Crit. Rev. Food Sci. Nutr.*, **55**, 740–749.

O'Hara, G. W., & Glenn, A. R. 1994. The adaptive acid tolerance response in root nodule bacteria and Escherichia coli. *Arch. Microbiol.*, **161**, 286–292.

O'Hara, G. W., Goss, T. J., Dilworth, M. J., & Glenn, A. R. 1989. Maintenance of Intracellular pH and Acid Tolerance in Rhizobium meliloti. *Appl. Environ. Microbiol.*, **55**, 1870–1876.

Pfaffl, W. M. 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.*, **29**, 2003–2007.

Puranamaneewiwat, N., Tajima, S., & Niamsup, H. 2006. Proteomic Analysis of Bradyrhizobium japonicum USDA110 in Acidic Condition. *Chiang Mai J. Sci.*, **33**, 335–345.

Quandt, J., & Hynes, M. F. 1993. Versatile suicide vectors which allow direct selection for

gene replacement in Gram-negative bacteria. *Gene*, **127**, 15–21.

Rhichardson, A. ., & Simpson, R. J. 1989. ACID-TOLERANCE OF RHIZOBIUM GROWING IN AN ACID SOIL. **21**.

Rice, A., Conado, A., & Alberto, T. H. 1982. *PERFORMANCE OF Rhizobium meliloti STRAINS SELECTED FOR LOW-pH TOLERANCE*. **948**, 941–948.

Sadowsky, M. J. 2006. Soil Stress Factors Influencing Symbiotic Nitrogen Fixation. *Nitrogen Fixat. Agric. For. Ecol. Environ.*, 89–112.

Sadowsky, M. J., Tully, R. E., Cregan, P. B., & Keyser, H. H. 1987. Genetic Diversity in Bradyrhizobium japonicum Serogroup 123 and Its Relation to Genotype-Specific Nodulation of Soybean. *Appl. Environ. Microbiol.*, **53**, 2624–2630.

Senouci-Rezkallah, K., Schmitt, P., & Jobin, M. P. 2011. Amino acids improve acid tolerance and internal pH maintenance in Bacillus cereus ATCC14579 strain. *Food Microbiol.*, **28**, 364–372.

Siegumfeldt, H., Rechinger, K. B., & Jakobsen, M. 2000. *Dynamic Changes of Intracellular pH in Individual Lactic Acid Bacterium Cells in Response to a Rapid Drop in Extracellular pH* (pp. 2330–2335).

Strous, M., Ormeño-Orrillo, E., Grunden, A. M., Sohlenkamp, C., Guerrero-Castro, J., & Lozano, L. 2018. Dissecting the Acid Stress Response of Rhizobium tropici CIAT 899. *Front. Microbiol. | Www.Frontiersin.Org*, **1**, 846.

Taylor, R. W., Williams, M. L., & Sistani, K. R. 1991. N2 fixation by soybean-Bradyrhizobium combinations under acidity, low P and high Al stresses. *Plant Soil*, **131**,

293–300.

Triplett, E. W., & Sadowsky, M. 1992. GENETICS OF COMPETITION FOR NODULATION OF LEGUMES. *Prospects*, 399–428.

Tusher, V. G., Tibshirani, R., & Chu, G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. U. S. A.*, **98**, 5116–5121.

Weidmann, S., Maitre, M., Laurent, J., Coucheney, F., Rieu, A., & Guzzo, J. 2017. Production of the small heat shock protein Lo18 from Oenococcus oeni in Lactococcus lactis improves its stress tolerance. *Int. J. Food Microbiol.*, **247**, 18–23.

Wu, C., Zhang, J., Chen, W., Wang, M., Du, G., & Chen, J. 2012. A combined physiological and proteomic approach to reveal lactic-acid-induced alterations in Lactobacillus casei Zhang and its mutant with enhanced lactic acid tolerance. *Appl. Microbiol. Biotechnol.*, **93**, 707–722.

Yan, D., Lin, X., Qi, Y., Liu, H., Chen, X., Liu, L., & Chen, J. 2016. Crz1p regulates pH homeostasis in Candida glabrata by altering membrane lipid composition. In *Applied and Environmental Microbiology* (Vol. 82, Issue 23).

Yang, Y., Kadim, M. I., Khoo, W. J., Zheng, Q., Setyawati, M. I., Shin, Y. J., Lee, S. C., & Yuk, H. G. 2014. Membrane lipid composition and stress/virulence related gene expression of Salmonella Enteritidis cells adapted to lactic acid and trisodium phosphate and their resistance to lethal heat and acid stress. *Int. J. Food Microbiol.*, **191**, 24–31.

CHAPTER 4

DEVELOPMENT OF RHIZOBIAL INOCULANTS WITH DUAL FUNCTION: NITROGEN FIXATION AND PHOSPHATE SOLUBILIZATION

ABSTRACT

Symbiotic association between soybean and *Bradyrhizobium japonicum* is of agricultural and ecological importance due to its contribution in soil nitrogen (N) fertility. The symbiotic nitrogen fixation and its benefit in legume-based cropping systems are well studied and understood. Inoculation of soybeans with *B. japonicum* is a promising strategy to maintain soil sustainability and to minimize adverse effects of synthetic N fertilizers. *B. japonicum* is widely used as an N biofertilizer and co-inoculation with other plant growth promoting rhizobia such as phosphate solubilizers has reported a remarkable increase in crop yield. In this study, the potential use of *B. japonicum* as a phosphorous (P) biofertilizer was investigated. P limitation is a major constraint in soybean cultivation because of its insoluble nature. Bacterial solubilization of inorganic phosphates through organic acid production plays a vital role in soil P mobilization. Gluconic acid is known as the widespread organic acid among phosphate solubilizing bacteria which is produced through the direct oxidation of glucose via glucose dehydrogenase (GDH) enzyme whose prosthetic group is pyrroloquinoline quinone (PQQ). *B. japonicum* USDA110 showed inorganic phosphate solubilization ability by dissolving $Ca_3(PO_4)_2$ up to 69.56 µg/ml of PO_4^{3-}. pH of the growth medium gradually dropped to final pH of 5.29 with increased solubilization of phosphate. Adding exogenous PQQ did not have a significant effect on its phosphate solubilization ability. Studies on reconstituted GDH enzyme activity measurements showed that *B. japonicum* produces

PQQ and its production is regulated by P availability and carbon source. Thus, the current study indicates that *B. japonicum* is a potential candidate to be used as a dual function biofertilizer with N fixation and P solubilization in agriculture.

INTRODUCTION

Phosphorous (P) is one of the most essential plant nutrients which has a structural and functional importance in growth and development of plants. It plays a vital role in major metabolic pathways and physiological processes in cells including photosynthesis, energy production, respiration, growth and development of roots, resistance to plant diseases, transmission of the genetic information (Kalayu, 2019; Khan *et al.*, 2009; Khan *et al.*, 2010). Interestingly, P is also involved in biological nitrogen fixation (BNF) in legumes (Kousa et al., 2005). P is a major component of macromolecules and cellular components such as nucleic acids, enzymes, co-enzymes, and phospholipids, which accounts for between 0.2 - 0.8% of plant dry weight (Sharma et al., 2013).

Deficiency of P retards plant growth ultimately reducing the crop yield. Unlike nitrogen (N), there is no large reservoir of atmospheric P which can be made biologically available for plant uptake. Plants absorb P from the soil solution either in the form of HPO_4^{2-} or $H_2PO_4^-$ anions based on soil pH. Although P is abundant in most of agricultural soils in both inorganic and organic forms, its bioavailability in the soil solution is limited because of its insoluble nature in soil minerals. Conventionally, soil P deficiency is overcome by adding synthetic P fertilizers. However, only a small amount of added phosphate fertilizer is absorbed by plant roots and a larger portion which is around 75-90% is precipitated with metal cation complexes in soil and become unavailable for plants soon after adding onto soil (Kalayu, 2019). The repetitive use of P fertilizers

leads to accumulation of inorganic phosphates in soil and creates adverse effects on environments including eutrophication and soil fertility depletion by disturbing the soil microbial community.

While being essential for plant growth and development, P plays an important role in symbiotic nitrogen fixation (SNF) in legumes. P deficiency directly interrupts SNF by limiting growth and persistence of rhizobia, and nodule formation and functioning (Isidra-Arellano et al., 2018). Besides, it has been reported that P deficiency affects nitrogenase enzyme activity by increasing oxygen concentrations within nodules (Isidra-Arellano et al., 2018). Hence, the relationship between P demand of host legume and the effective symbiosis is of prime importance in legume-based cropping systems. P naturally exists in soils as insoluble inorganic and organic reserves. Soil microorganisms have a significant contribution towards the soil P dynamics where they facilitate the mobilization of P among different pools of soil P.

A multitude of soil microorganisms including bacteria, fungi, actinomycetes and algae possess mineral phosphate solubilization and mineralization ability which makes them possible candidates as a P biofertilizer. Among them are bacteria greatly important because solubilization of mineral phosphate is a notable property of soil bacteria which enhances plant P nutrition. *Pseudomonas* spp., *Agrobacterium* spp. and *Bacillus circulans* are well studied soil bacteria with P mobilization ability (Babalola & Glick, 2012). The greatest number of P solubilizers are reported in genus *Bacillus* followed by *Pseudomonas*. Additionally, a number of strains of *Azotobacter, Burkholderia, Enerobacter, Erwinia, Kushneria* (Zhu et al., 2011)*, Paenibacillus, Ralstonia, Rhizobium, Rhodococcus, Serratia, Bradyrhizobium, Salmonella, Sinomonas* and *Thiobacillus* (Sharma et al., 2013) are known to be involved in P solubilization and mineralization. Phosphate solubilizing soil rhizobacteria deploy a variety of methods to make insoluble inorganic phosphates available for plants. The principal mechanism for P solubilization is the production of mineral

dissolving compounds such as organic acids, siderophores, protons, hydroxyl ions, and CO_2 which cause lowering of soil pH. Additionally, they dissolve inorganic phosphates through chelation and mineralization.

Solubility of inorganic phosphates is accelerated by decline of soil pH. Phosphate solubilizing bacteria (PSBs) achieve this by production of organic acids or by release of protons (Kumar et al., 2018; Walpola & Yoon, 2012). Organic acids are mostly produced during the metabolism of organic carbon sources by bacteria via direct oxidation or fermentation in the periplasmic space. Organic acid mediated phosphate solubilization is widespread among PSBs and they release different types of organic acids while gluconic acid and 2-ketogluconic acid are the ubiquitous. Gluconic acid is the key organic acid produced by PSBs such as *Pseudomonas* sp., *Erwinia herbicola*, *Burkholderia cepacia* (Rodríguez & Fraga, 1999) while 2-ketogluconic acid is found in *Rhizobium leguminosarum*, *Sinorhizobium meliloti,* and *Bacillus firmus*. The well-studied mechanism of bacterial phosphate solubilization is through secretion of gluconic acid (Goldstein, 1995) which is produced through direct oxidation of glucose by a glucose dehydrogenase (GDH) enzyme that requires the redox prosthetic group (4,5-dihydro-4,5-dioxo-1Hpyrrolo-[2,3-f]quinoline-2,7,9-tricarboxylic acid or pyrroloquinoline quinone (PQQ) (Duine et al., 1979; Naveed, 2016). PQQ, also known as methotaxin, is a water soluble, heat stable tricyclic ortho-quinone (Puehringer et al., 2008), which functions as the redox cofactor for various bacterial dehydrogenases such as methanol, ethanol and glucose dehydrogenases and the first to be found in this cofactor family. Structural data from PQQ biosynthesis-related studies show that PQQ is derived from the two amino acids glutamate and tyrosine. Bacterial production of PQQ is the primary source, since neither plant nor animals can synthesis PQQ. Many bacteria possess the genes *pqqABCDEF/G* required for the PQQ production. PQQ also plays key roles in animals and

plants. In animals, PQQ repairs neurological injury, acts as an anti-melanogenic agent against hyper pigmentation, immunity booster, treatment for insomnia, cancer fighter, liver fibrogenesis, signal transduction via mitochondrial biogenesis, protects from cardiac disease, and as a vitamin supplement. In plants, PQQ involves in phosphate solubilizing activities, plant growth promotion, antifungal activities, and induced systemic resistance. Recently, PQQ has shown multiple applications in modern technology as a bio-electro catalysis, conductive polymeric fibers in polymer technology, and charge transfer mediated nanoparticles. While being the sole source of natural PQQ, microbes have a multitude of advantages as a growth stimulant, antibiotics, energy transduction, ATP synthesis during oxidation, intracellular signaling and DNA repair.

GDH is a quinoprotein that requires PQQ and possesses binding sites for Ca^{2+}, ubiquinone and for substrate glucose for its activity. Two types of GDH enzymes have been identified, GDH A and GDH B based on its location within the cell. GDH A, a membrane-bound enzyme (m-GDH) is more prominent and reported in *Acinetobacter calcoaceticus, Pseudomonas aeruginosa, Gluconobacter suboxydans, Klebsiella aerogenes Acinetobacter lwoffi and Escherichia coli.* GDH B is soluble (s-GDH) and is reported only from *Acinetobacter calcoaceticus* (Cleton-Jansen et al., 1990). The presence of a PQQ-dependent GDH and inorganic phosphate solubilization capabilities have been reported in rhizobia as well (Abd-Alla, 1994; Halder & Chakrabartty, 1993). However, some rhizobia such as *Rhizobium legumiosarum* and *Sinorhizobium meliloti* and several strains of *Escherichia coli* were able to produce gluconate from glucose in the presence of exogenous PQQ, indicating that they are unable to synthesize PQQ and possess the apo-GDH. In contrast, some bacteria such as *Deinococcus radiodurans* synthesize PQQ but, do not exhibit phosphate solubilization capabilities (Khairnar et al., 2003). Therefore, PQQ-dependent GDH activity, PQQ

biosynthesis, and inorganic phosphate solubilization are highly variable and widespread among bacteria, specifically rhizobacteria.

In this study we are trying to develop a dual function rhizobium as a biofertilizer to provide plants with both N and P simultaneously. *Bradyrhizobium japonicum* USDA110, nitrogen fixing symbiont of soybean is a commercially well-established N biofertilizer over several decades. Interestingly, it has shown a PQQ-dependent holo GDH enzyme activity (Boiardi et al., 1996), creating an avenue for further research on its potential to dissolve inorganic phosphate and utilization as a P biofertilizer. Specifically, *B. japonicum* USDA110 possess the PQQ operon which was induced in response to desiccation stress to protect cells reducing oxidative stress (Cytryn et al., 2007). A few studies have demonstrated the mineral phosphate solubilization properties of *B. japonicum* species (Abd-Alla, 1994). However, contribution of PQQ in mineral phosphate solubilization and mechanisms employed by *B. japonicum* USDA110 is still unknown.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Bradyrhizobium japonicum* USDA110 was grown aerobically at 30°C with shaking at 200rpm in Bergersen's minimal medium (BMM) at pH 6.8 supplemented with 0.4% glycerol as the sole carbon source which contains 300 mg of K_2HPO_4, 300 mg of KH_2PO_4, 500 mg of NH_4NO_3, 100 mg of $MgSO_4.7H_2O$, 50 mg of NaCl, 1 ml of 1000X vitamin stock contains 20 mg of riboflavin, 20 mg of p-amino benzoic acid, 20 mg of nicotinic acid, 20 mg of biotin, 20 mg of thiamine HCl, 20 mg of pyridoxine HCl, calcium pantothenate and 120 mg of inositol dissolved in 0.5 M Na_2HPO_4 (pH 7.0), and 1 ml of 1000X trace elements stock contains 5g of $CaCl_2$, 145 mg of H_3BO_3, 125 mg of $FeSO_4.7H_2O$, 70 mg of $CoSO_4.7H_2O$, 5 mg of $CuSO_4.7H_2O$, 4.3 mg of $MnCl_2.4H_2O$, 108 mg of $ZnSO_4.7H_2O$, 125 mg of $NaMoO_4$ and 7 g of nitrilotriacetate (pH 5.0) per 1 L. To study the inorganic phosphate solubilization ability of the *B.*

japonicum USDA110, BMM media was prepared without K_2HPO_4, and KH_2PO_4 to create a zero dissolved phosphorous medium. 1000X vitamin stock was prepared using distilled water instead of 0.5 M Na_2HPO_4 to dissolve the ingredients. Tri calcium phosphate $Ca_3(PO_4)_2$ was added to the zero dissolved phosphorous BMM as the sole source of phosphorous. To validate the PQQ quantification protocol, *E. coli* DH5α and *Pseudomonas putida* KT2440 strains grown in LB medium which contains 10g of tryptone, 5g of yeast extract and 10g of NaCl (pH 7.0) were used as the negative and the positive control, respectively.

Selecting the PQQ treatment condition. To study the effect of exogenous PQQ on the growth of *B. japonicum* USDA110, cultures were grown aerobically at 30°C in BMM (pH 6.8) with different concentrations (1, 3, 10 and 100 µM) of PQQ. Growth rates were compared with that of the culture without PQQ which served as the control. Then the concentration range was narrowed down to 1-10 µM PQQ to select the PQQ treatment condition.

Evaluation of *B. japonicum* for inorganic phosphate solubilization ability. The inorganic phosphate solubilization ability of *B. japonicum* USDA110 was evaluated by growing the bacterium in zero dissolved phosphorous liquid medium with insoluble tricalcium phosphate as the sole phosphate source and measuring the solubilized phosphorous concentration in the culture filtrate over time. One hundred milliliter of BMM broth (pH 6.8) containing 0.4% glycerol as the sole carbon source was added into 250ml flasks. Insoluble $Ca_3(PO_4)_2$ was added as the only source of phosphorous to the medium at the concentration of 1.08 gL^{-1}. Each flask was inoculated with a 100 µl of *B. japonicum* USDA110 grown in 10 ml of BMM medium at 30°C with shaking at 200 rpm up to mid-exponential growth. To study the effect of exogeneous PQQ on the phosphate solubilization efficiency of the bacterium, PQQ was added to the medium at a concentration of 10 µM. Uninoculated BMM media with $Ca_3(PO_4)_2$ was used as the negative control of the experiment.

Three replicates were used for each treatment. Both inoculated and uninoculated flasks were incubated at 30℃ with shaking at 200 rpm for 12 days. pH of the medium, cell density and dissolved phosphorous concentration were measured in every 24 h by taking 3.5 ml from each flask. Of this 3.5 ml volume 2.5 ml was used to measure pH using a benchtop pH meter (Fisher Scientific) and 500 µl was used for measuring cell density at OD_{600nm}. OD_{600nm} was measured spectrophotometrically (BioSpec Mini) after adding same volume of 3.7% HCl to dissolve remaining $Ca_3(PO_4)_2$ in the medium (An & Moe, 2016). The remaining 1 ml was centrifuged at 14,500 rpm using micro centrifuge (Eppendorf mini spin) for 30 min. and the supernatant was filter sterilized through 0.22µM filters (Fisher Scientific). 500 µl of the sterilized filtrate was diluted up to 1 ml with sterilized distilled water and dissolved inorganic phosphate concentration was measured using the Vanado-molybdate method (Chen et al., 1956). Briefly, 1 ml of freshly made Chen's reagent, containing 10% (w/v) ascorbic acid, 3M sulfuric acid, 2.5% (w/v) ammonium molybdate and sterilized distilled water in 1:1:1:2 ratio was added to the same volume of diluted filtrate (1 ml) in a 15 ml centrifuge tube and mixed well by vortexing. Then reaction mixture was incubated at 37℃ for 1 h and let it cool down for 5 min. The absorbance at 880 nm was measured spectrophotometrically using 1 ml of the reaction mixture on BioSpec Mini spectrophotometer after vortexing thoroughly. Dilution of the reaction mixture before absorbance measurements was done as needed. The inorganic phosphate concentration in the sample was determined by a standard curve constructed using KH_2PO_4 and expressed as µg of PO_4^{3-} ml^{-1}.

Glucose dehydrogenase enzyme activity assay. Glucose dehydrogenase (GDH) enzyme (PQQ dependent; from native microorganism, EC 1.1.5.2) was purchased from CREATIVE ENZYMES (New York, USA). Holo-GDH enzyme (1 mg) was dissolved in 1 ml of 0.1 M phosphate buffer (containing 1 mM ethylenediamine-N, N, N', N'-tetra acetic acid (EDTA) and 2

M KBr; pH 7.3) and placed in a slide-A-Lyzer 20K MWCO MINI Dialysis Device (ThermoFisher). For inactivation of the GDH, dialysis was carried out at 4˚C for 30 h. The dialysis device containing the enzyme solution was placed in a 15ml centrifuge tube containing 15 ml of 0.1 M phosphate buffer and shaken (speed: level 2) on a platform shaker (VWR) at 4˚C for 6 h. Then the buffer was renewed and dialyzed again for 24 h in the same buffer to remove GDH bound PQQ. Then the resultant solution was dialyzed against 20 mM 3- morpholino-propanesulfonic acid (MOPS) buffer (pH 7.0) at 4˚C for 30 h (Kato et al., 2018).

GDH enzyme activity was measured using a colorimetric assay involving nitrotetrazorium blue (NTB, Fisher) and phenazine methosulphate (PMS, Fisher) according to the enzyme manufacture's protocol. The enzyme activity is measured using the appearance of diformazan produced from the reduction of NTB by reduced PMS through PQQ- dependent GDH mediated oxidation of D-glucose. Diformazan production was spectrophotometrically quantified at 570 nm on microplate reader (BioTek Synergy 2). One unit of the enzyme is defined as the formation of one-half micromole of diformazan per minute under the reaction conditions described below. The enzyme reaction mixture contains 0.9 ml of 1M D-glucose, 25.5 ml of 50 mM PIPES-NaOH (pH 6.5), 2.0 ml of 3 mM PMS and 1.0 ml of 6.6 mM NTB. The above reaction mixture was freshly prepared in a brownish bottle and stored on ice until use. Then 3.0 ml from the reaction mixture was taken to a 15 ml centrifuge tube and equilibrated at 37°C for 5 min. Prepared apo-GDH enzyme was diluted to 0.1-0.8 U/ ml (0.5 U/ ml) with GDH enzyme diluent of 50 mM PIPES-NaOH buffer (pH 6.5) containing 1 mM $CaCl_2$, 0.1% Triton X-100 and 0.1% BSA on ice before the assay. Reconstitution of the holo-enzyme was done before the assay by incubating 100 µl of apo-GDH with 25 µl of PQQ standards with varying concentrations or culture supernatants at 25°C for 30 min.100 µl of the reconstituted holo-enzyme solution was added to the reaction mixture in

the centrifuge tube and gently mixed by pipetting. Increase of optical density at 570 nm was recorded against water for 5 min. in a microplate reader (BioTek Synergy 2) thermostated at 37°C. Optical density change per minute was calculated. At the same time the blank rate was measured by the same method using the same reaction mixture except the enzyme diluent was added instead of the enzyme solution. The same assay was conducted with the original holo-enzyme and the prepared apo-enzyme to compare the activity of reconstituted holo-enzyme using the same reaction conditions. Enzyme activity was calculated by using the following formula:

$$Volume\ activity\ (U/ml) = \frac{\Delta OD/min\ (\Delta OD\ test - \Delta OD\ blank) \times Vt \times\ df}{20.1 \times 1.0 \times Vs}$$

Vt: Total volume (3.1 ml)

Vs: sample volume (0.1 ml)

20.1: Half a millimolar extinction coefficient of diformazan (cm^2/ 0.5 micromole)

1.0: Light path length (cm)

Df: Dilution factor

PQQ bioassay. PQQ concentrations in the culture supernatant were determined using the enzyme assays conducted with the apo-GDH enzyme. This experiment is based on the fact that the apo-GDH enzyme is inactive due to lack of bound PQQ, and it shows the activity only after adding exogenous PQQ. A standard curve was generated from assays with the reconstituted holo-GDH enzyme with PQQ standards of varying concentrations. Culture supernatants of *B. japonicum* USDA110 grown under different conditions in BMM medium, *E. coli* DH5α and *Pseudomonas putida* KT2440 grown in LB medium were obtained by centrifugation at 14,500 rpm for 30 min. (Eppendorf Mini Spin). Filter sterilized (0.22 m, Fisher Scientific) supernatant was incubated with

apo-GDH enzyme and then used to measure enzyme activity as described above. The standard curve was used to determine the PQQ concentrations in culture supernatants in ng/ ml according to the enzyme activity.

RNA isolation. Total RNA was isolated from *B. japonicum* USDA110 cultures grown in BMM treated with 10 µM PQQ and control without PQQ at mid-exponential phase. Fifty milliliters of *B. japonicum* USDA110 culture was grown at 30°C with shaking at 200 rpm until it reaches early log phase. Then exogenous PQQ was added at the concentration of 10 µM and incubated until it reaches mid log phase. Three biological replicates were used for each treatment, and culture grown without PQQ was used as the control. Harvesting of cells for RNA extraction was performed as previously described (Jeon et al., 2011). Cultures were condensed by centrifugation for 20 min at 4°C and 4,000 rpm in a fixed angle rotor (Eppendorf 5401). The cell pellet was then collected by decanting the supernatant. RNA isolation followed by using hot phenol method as described previously (Taw et al., 2015). The RNeasy mini kit (Qiagen) and RNase-free DNase (Qiagen) was used to purify the isolated RNA according to manufacturer's protocol. RNA quantity was calculated using NanoDrop device (Thermo Scientific) and RNA quality was checked via gel electrophoresis.

RNA sequencing and transcriptomic analysis. The transcriptomes of *B. japonicum* USDA110 grown without PQQ and treated with 10 µM PQQ were analyzed and compared by submitting total RNA samples to genomic Sequencing and Analysis Facility, center for Biomedical Research at The University of Texas at Austin (GSAF-UT Austin). Library preparation with ribosomal RNA depletion and sequencing were done according to the guidelines of GSAF-UT Austin using run type single end reads (SR 75) on NextSeq 500 platform with a sequencing depth

of 6 million reads per sample. Aligned reads to *B. japonicum* USDA 110 reference genome was analyzed using DESeq package (Love et al., 2014)**.**

RESULTS

Effect of different concentrations of PQQ on *B. japonicum* USDA110 growth. Growth curve studies of *B. japonicum* USDA110 were conducted with different concentrations of exogenous PQQ to find out the optimum level of PQQ that should be used for the downstream assays. Generation time of the bacterium grown under each concentration of PQQ was compared with that of the control grown without PQQ. Generation time of the control, 1 µM, 3 µM, 10 µM and 100 µM is calculated as 8.55 h, 11.12 h, 13.22 h, 8.08 h and 9.09 h, respectively. According to the results, 10 µM PQQ was selected as the optimum level of PQQ that shows closest growth rate to the control culture (Fig.4-1).

Effect of availability of phosphorous and PQQ on *B. japonicum* USDA110 growth. To study the effect of available phosphorous on the growth of *B. japonicum* USDA110, cell density was monitored in the cultures grown in BMM with dissolved phosphorus (K_2HPO_4 and KH_2PO_4) and BMM with insoluble inorganic phosphate source ($Ca_3(PO4)_2$). Effect of exogenous PQQ was tested by adding 10µM PQQ to each culture. Data showed that the bacterium is growing faster when dissolved phosphorous (K_2HPO_4 and KH_2PO_4) is available in the growth medium irrespective of the presence of exogenous PQQ. However, the presence of exogenous PQQ was found to be enhancing growth rate in phosphorous deprived BMM than the phosphorous rich BMM. When the bacterium had optimum concentration of dissolved phosphorus (K_2HPO_4 and KH_2PO_4) available in the growth medium, no significant difference was observed in growth rate or cell density with or without PQQ (Fig.4-2).

Inorganic phosphorus solubilization along with pH changes upon PQQ. To study the ability of *B. japonicum* USDA110 to solubilize inorganic phosphate, a zero dissolved phosphorous medium was prepared by replacing K_2HPO_4 and KH_2PO_4 with $Ca_3(PO4)_2$ in BMM. To grow in the BMM with insoluble phosphate, *B. japonicum* USDA110 must dissolve $Ca_3(PO4)_2$ to fulfill their phosphorous requirement. Dissolved PO_4^{3-} concentration in the culture supernatant was measured over time with its growth. The initial dissolved phosphorus concentration in the medium at beginning of the experiment (at 0 time point) upon addition of $Ca_3(PO4)_2$ was 0.43 µg/ ml. Dissolved PO_4^{3-} concentration in BMM culture supernatant began to increase after 3 days of incubation and drastically increased over next 6 days during the experimental period in both cultures with and without exogenous PQQ reaching the maximum of 69.56 µg/ml and 68.97 µg/ml respectively which is not statistically significant ($P < 0.05$). The decline of pH in culture with exogenous PQQ was significantly ($P < 0.05$) faster than that without PQQ up to day 4 and then it was slightly declined in a decreasing rate than the control. Dissolved PO_4^{3-} concentration (0.43 µg/ml) and pH (pH 7~7.3) in uninoculated control were constant at their initial values over time (Fig. 4-3).

GDH enzyme activities and PQQ production. Holo-GDH purchased from the manufacturer was converted to its apo form which is inactive through dialysis by removing GDH bound PQQ. Activity of holo-GDH and apo-GDH were measured to be 0.146 U/ml and 0.002 U/ml, respectively. Activity of the reconstituted holo-GDH enzyme pre-incubated with known PQQ standard concentrations was used to generate a standard curve between PQQ concentration and the enzyme activity. Then, the activity of apo-GDH incubated with culture supernatant of *B. japonicum* USDA110 grown under different growth conditions was assessed to quantify the concentration of PQQ in the culture supernatants. The highest activity of the reconstituted holo-

GDH was reported when the bacterium was provided with BMM with its optimum level of dissolved phosphorous and 0.4% glycerol as the sole Carbon(C) source. Significantly low enzyme activity ($P < 0.05$) was shown when the apo-GDH was incubated with culture supernatant from the bacterium grown with glucose as the sole C source. There was no significantly different enzyme activity shown when the cultures were grown with insoluble phosphate $(Ca_3PO_4)_2$ irrespective of the C source. Concentration of PQQ present in the culture supernatant was statistically significant based on the C source. The highest concentration was shown in the supernatant grown in BMM with dissolved phosphorous and 0.4% glycerol.

Transcriptomic analysis with PQQ treatment. RNA sequence data analysis showed that most of the genes that were significantly up and down regulated in the presence of exogenous PQQ are genes coding proteins with hypothetical functions. Gene *bll2752* coding for glycosyl transferase family 4 protein which may involve either in polysaccharide synthesis or inorganic phosphate transfer was induced 2.99 folds and stress response related Csbd family protein coding gene *blr1496* was up regulated 2.44 times. Additionally, efflux RND transporter periplasmic adaptor subunit coding gene *blr4936* which may be associated with pumping out antibiotics or other chemical and or protons from the cells was induced 2.41folds. Among the down regulated genes, *blr7037* coding chaperone NapD, *bll3998* coding (NAD-dependent succinate-semialdehyde dehydrogenase, *blr1289* coding oleate hydratase, *bll6069* for universal stress protein, *bll5655* for alcohol dehydrogenase AdhP and *blr2611* coding helix-turn-helix domain-containing protein were suppressed in 30.53, 30.40, 28.18, 24.36, 18.06 and 17.89 folds (Table 4-1, Table 4- 2).

DISCUSSION

Growth curve studies done with different concentrations of PQQ in this study revealed that adding minute concentrations of PQQ does not have a positive impact on the growth of *B. japonicum* USDA110. In contrast, adding higher concentrations of PQQ (> 10 µM) reduced the growth rate of the bacterium than that of the control which was grown without exogenous PQQ. In this study concentration of 10 µM was identified as the optimum concentration of PQQ that benefits the growth of *B. japonicum* USDA110. PQQ was first discovered in 1979s as a redox cofactor of bacterial dehydrogenases which involve in organic acid production. PQQ-dependent dehydrogenases are widespread among bacteria and biosynthesis of PQQ is confined in prokaryotes. Structural and functional profile of PQQ as a coenzyme is well characterized and found to play a vital role in bacterial energy transduction. Additionally, PQQ exhibits interesting properties as a dietary supplement to improve human health and plant growth promoter (Ferri et al., 2011; Naveed, 2016). In bacteria PQQ also acts as a growth stimulant. Two types of growth stimulation by PQQ have been identified. In the first type of growth stimulation growth rate and production of cells increases without any remarkable reduction of lag phase. In the second type of growth stimulation a noticeable reduction of the lag phase is observed without affecting the subsequent growth rate at the exponential phase and total growth of cells at the stationary phase. However, PQQ is not an essential growth factor and bacteria can grow even without PQQ. Even though the reduction of lag phase is associated with the externally added PQQ, the optimum concentration of PQQ that results in reduced lag phase and increased growth is totally dependent on the strain of bacteria. Trace amounts of PQQ is known to promote bacterial growth and adding of too much PQQ inhibits bacterial growth and reduction of lag phase (Ameyama et al., 1984). According to the results of this study, *B. japonicum* USDA110 show the second type of growth

stimulation in response to higher concentrations of PQQ in which a marked reduction of lag phase is not observable.

A number of strains of *Rhizobium* and *Bradyrhizobium* species are known as phosphate solubilizers (Antoun et al., 1998; Halder & Chakrabartty, 1993). Phosphate solubilizing ability of *B. japonicum* USDA110 is understudied and in some cases, it has been reported as a non-phosphate solubilizing species (Fernández et al., 2005). However, its ability to solubilize $Ca_3(PO4)_2$ and rock phosphate in broth was demonstrated in a study where 88.74 mgL^{-1} and 39.21 mgL^{-1} of P was dissolved respectively (López-ortega et al., 2013). Further, the results of our experiment conducted with $Ca_3(PO4)_2$ showed that *B. japonicum* USDA110 dissolve inorganic phosphates up to 68.97 µg/ml of PO_4^{3-}. However, the dissolved P concentration reported in this study is comparatively less than that of the previous reported data. This difference might be due to the variation between the duration of the experiment conducted. Our current study reports the dissolved P in culture supernatant only after 9 days while the previous study was done for 12 days. Even in this study we noticed an increase of P solubilization over time until 12 days for which data is not shown. Ability of *B. japonicum* USDA110 to dissolve inorganic phosphates was found to be independent from the presence of exogenous PQQ. Adding 10 µM PQQ did not significantly increased the concentration of dissolved P in the culture supernatant. It is an indication of either its ability to biosynthesize PQQ or to solubilize phosphates in an alternative route without needing of PQQ. pH measurements of the culture medium showed that pH of medium was declined significantly with increase of phosphate solubilization. pH of the medium dropped up to pH 5.07 from pH 7.00. This finding is in accordance with the previously reported data of *B. japonicum* USDA110 phosphorus solubilization with $Ca_3(PO4)_2$ which recorded a final pH of 5.29 (López-ortega et al., 2013).

Innate production of PQQ by *B. japonicum* USDA110 was revealed in the enzyme activity measurement studies conducted using apo-GDH and culture supernatants. PQQ production and holo-GDH activity of *B. japonicum* is not explicitly stated and needs thorough investigations. The presence of the PQQ-dependent GDH is highly variable among the strains of *Rhizobium* and *Bradyrhizobium*. Surprisingly, some strains of bacteria including rhizobia such as *E. coli* (Hommes et al., 1985), *R. leguminosarum* and *Sinorhizobium meliloti* (Bernardelli et al., 2001) possess the apo-form of GDH and do not show the capability of PQQ production. In these bacteria, reconstitution of active holo-GDH is possible when exogenous PQQ is added to the growth medium (Boiardi et al., 1996). However, presence of PQQ-linked GDH either in holo or apo form and production of PQQ is strain dependent. *B. japonicum* USDA110 has shown holo-GDH enzyme activity but gluconate production (Boiardi et al., 1996). This may be due to subsequent metabolism of gluconate upon production. In many bacteria, PQQ-dependent GDH is responsible for direct oxidation of glucose into gluconate or gluconic acid which is mainly involved in inorganic phosphate solubilization. *Rhizobium* and *Bradyrhizobium* species metabolize glucose through the Entner Doudoroff (ED) and pentose phosphate pathway. Gluconate is mainly metabolized via ED pathway and the ketogluconate (KG) pathway (Stowers & Gerald, 1984; Keele et al., 1970; Stowers, 1985). Bacterial production of PQQ is directly associated with the growth conditions such as availability of carbon source and phosphorous. It has been found that GDH activity and PQQ production of *Pseudomonas putida* KT2440 are optimum when the bacterium is provided with glucose as the sole carbon source and under P starvation (An & Moe, 2016).

Activity assessment of reconstituted holo-GDH with *B. japonicum* USDA110 culture supernatants indicated that the enzyme activity and PQQ biosynthesis is maximum when it has optimum level of dissolved P as K_2HPO_4 and KH_2PO_4 (4.5 mM) and glycerol as sole carbon

source. Reconstituted enzyme activity and PQQ production were significantly low when glucose is provided as the sole carbon source with optimum level of dissolved P as K_2HPO_4 and KH_2PO_4. This difference could be directly attributed to the amount of PQQ produced and bacterial GDH activity. When the bacterium is provided with glycerol and the optimum P needed for growth, the activity of bacterial GDH goes down and a considerable portion of the produced PQQ is exuded to the culture medium. There was no significant difference of the reconstituted enzyme activity and the PQQ production when the bacterium is grown in P deprived medium irrespective of the carbon source. Bacterial GDH activity increases to produce gluconic acid and to induce inorganic phosphate solubilization under zero dissolved P conditions. Therefore, produced PQQ is being utilized in the gluconic acid production pathway and amount of PQQ detected in the culture supernatant become reduced.

Transcriptomic analysis did not show much of a significant difference among the number and variety genes that were up and down regulated in response to external PQQ. That may be due to innate production of PQQ inhibiting the utilization of PQQ from outside of the cells, so no genes are specifically induced. Presence of exogenous PQQ may be a stress factor for the bacteria since some stress related proteins such as Csbd family proteins were up regulated. Adding too much of PQQ may have inhibitory effects on bacterial growth (Ameyama et al., 1984).

Findings of this study will open a new avenue for sustainable soil fertility improvement with the dual function biofertilizer.

Table 4-1. List of genes up regulated in response to exogenous PQQ (10 µM)

Gene name	Function	Fold change
blr7475	Phage holin family protein	3.09
blr4100	hypothetical protein	3.03
bll2752	glycosyl transferase family 4 protein	2.99
blr1469	DUF2865 domain-containing protein	2.87
bll7642	DUF1236 domain-containing protein	2.85
blr4223	SH3 domain-containing protein	2.75
blr3169	DUF1236 domain-containing protein	2.52
bsr2400	hypothetical protein	2.48
bll6467	hypothetical protein	2.46
blr1496	CsbD family protein	2.44
bll6993	hypothetical protein	2.42
blr4936	efflux RND transporter periplasmic adaptor subunit	2.41
blr5214	hypothetical protein	2.40
bll1465	hypothetical protein	2.39

Table 4-2. List of genes down regulated in response to exogenous PQQ (10 µM)

Gene name	Function	Fold change
blr6074	CBS domain-containing protein	-55.49
blr7345	hypothetical protein	-39.66
blr0497	Hypothetical protein	-31.41
blr7037	chaperone NapD	-30.53
bll3998	NAD-dependent succinate-semialdehyde dehydrogenase	-30.40
blr1289	oleate hydratase	-28.18
bll6069	universal stress protein	-24.36
bsl7992	DUF2933 domain-containing protein	-22.04
blr4114	DUF485 domain-containing protein	-20.44
bsr6066	hypothetical protein	-18.76
bll5655	alcohol dehydrogenase AdhP	-18.06
blr2611	helix-turn-helix domain-containing protein	-17.89
bll7551	hypothetical protein	-17.25

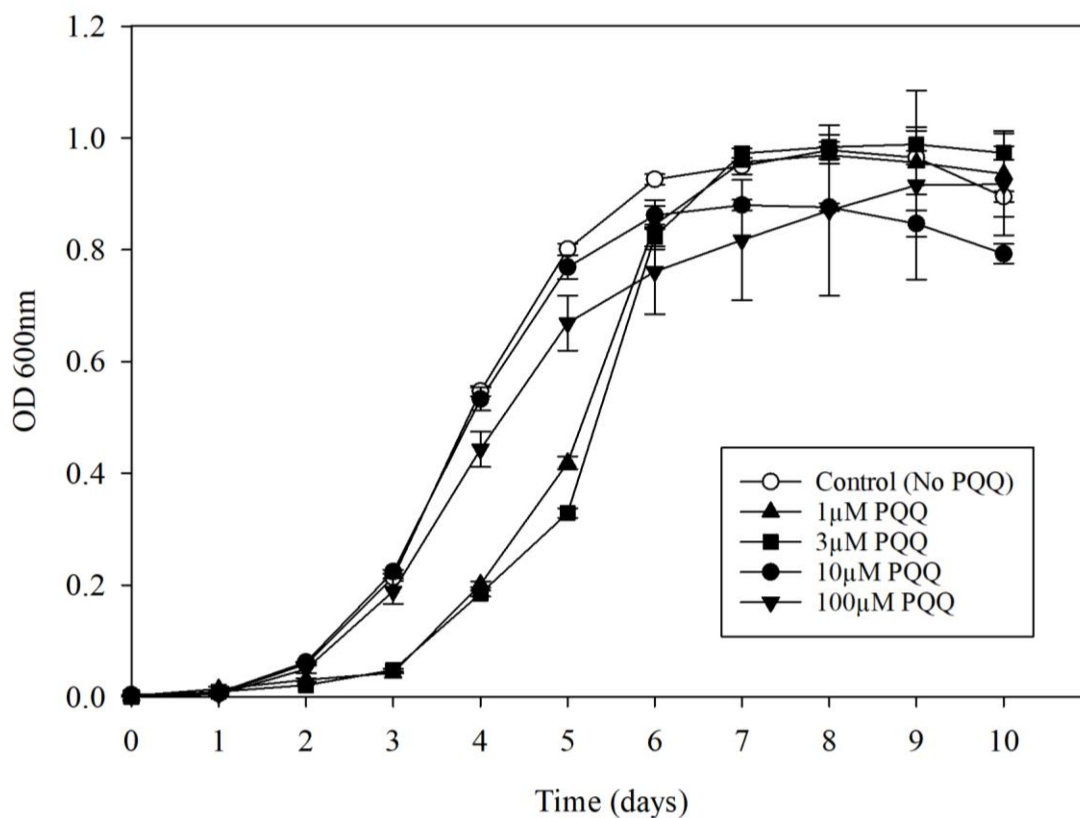

Figure 4-1. Growth of *B. japonicum* USDA110 in Bergesen's minimal medium (BMM) supplemented with 0.4% glycerol as sole carbon source with different PQQ concentrations. Each time point represents an average OD of three replicates with standard deviation. Symbols: ○, *B. japonicum* USDA110 control without PQQ; ▲, 1 µM PQQ; ■, 3 µM; ●, 10 µM PQQ; ▼, 100 µM PQQ.

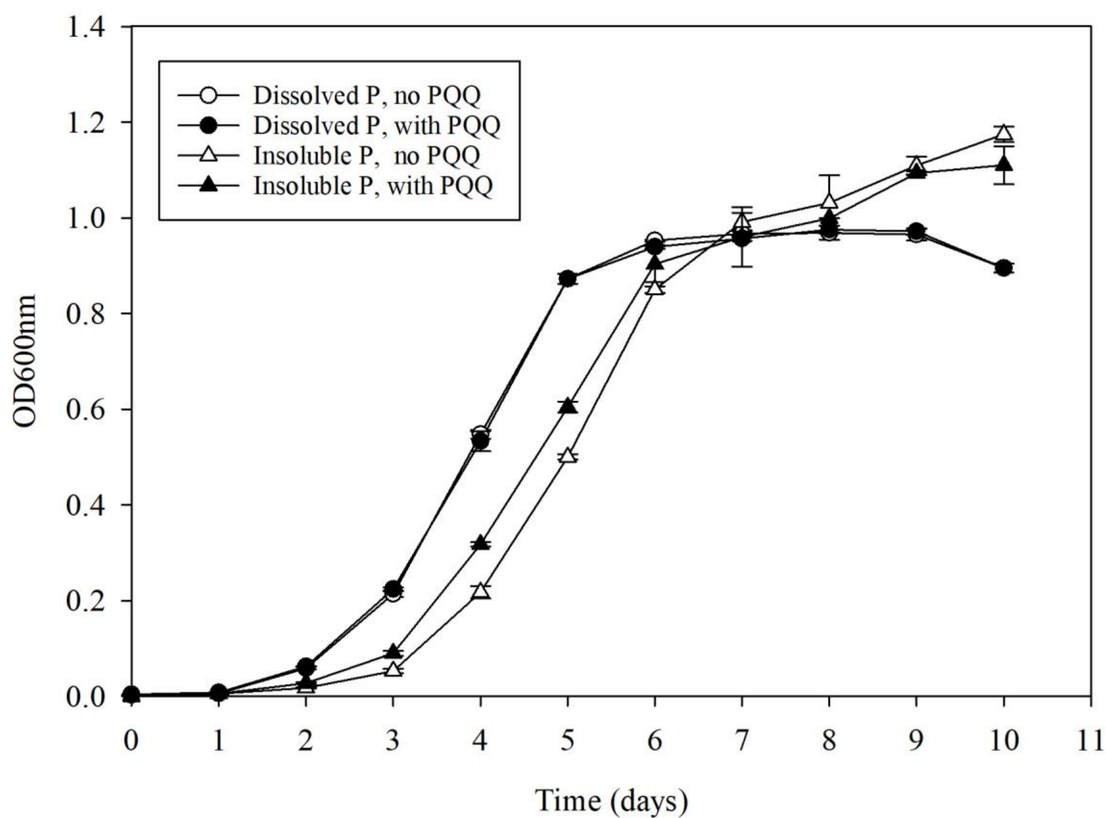

Figure 4-2. Growth of *B. japonicum* USDA110 in Bergesen's minimal medium (BMM) supplemented with 0.4% glycerol as sole carbon source with dissolved phosphorous and without dissolved phosphorous; tri calcium phosphate added as sole source of phosphate. Each time point represents an average OD of three replicates with standard deviation. Symbols: ○, dissolved P (KH_2PO_4 and K_2HPO_4) without PQQ; ●, dissolved P (KH2PO4 and K2HPO4) with 10 µM PQQ; Δ, insoluble P $(Ca_3PO_4)_2$ without PQQ; ▲, insoluble P (Ca3PO4)2 with 10 µM PQQ.

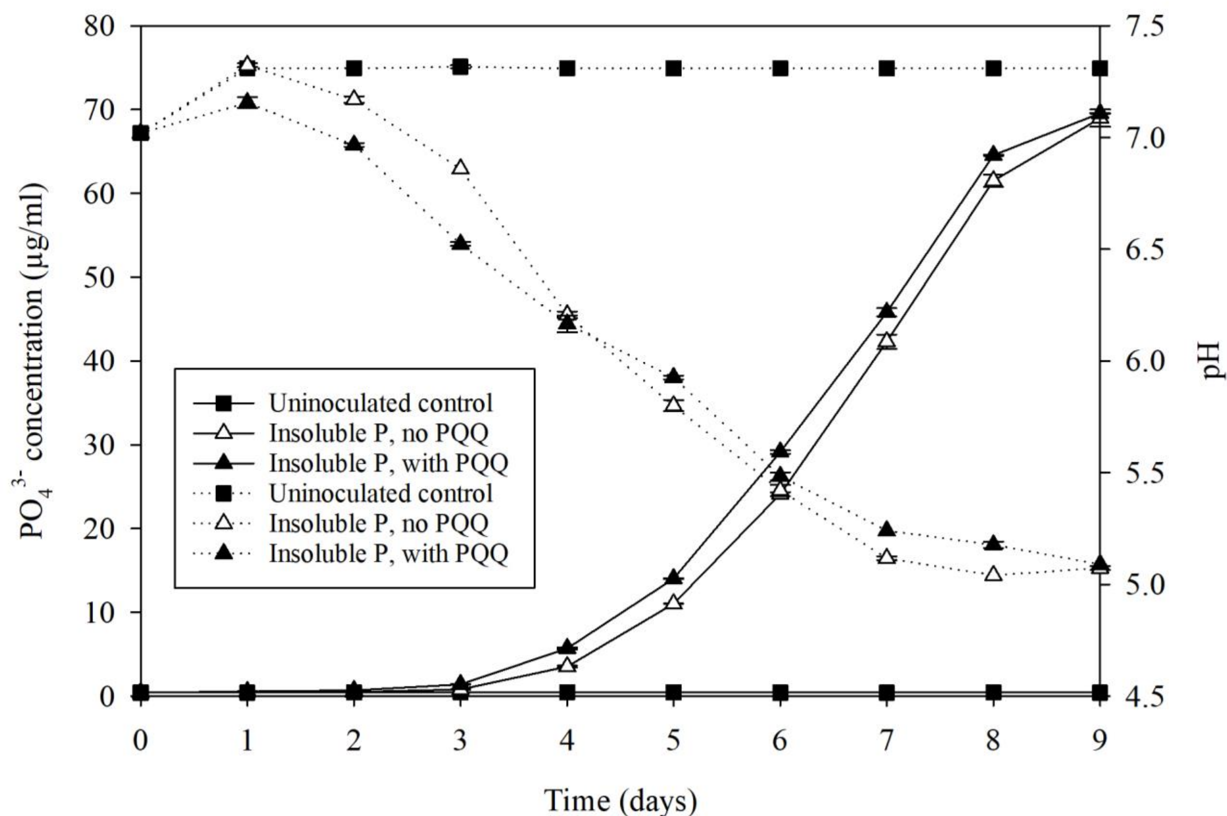

Figure 4-3. pH changes and solubilized phosphorus concentration in *B. japonicum* USDA110 culture in Bergesen's minimal medium (BMM) supplemented with 0.4% glycerol as sole carbon source with tri calcium phosphate as sole source of phosphorous. Each time point represents an average pH and concentration of $PO_4{}^{3-}$ of three replicates with standard deviation. Symbols: ■, uninoculated control; Δ, insoluble P $(Ca_3PO_4)_2$ without PQQ; ▲, insoluble P $(Ca_3PO_4)_2$ with 10 µM PQQ.

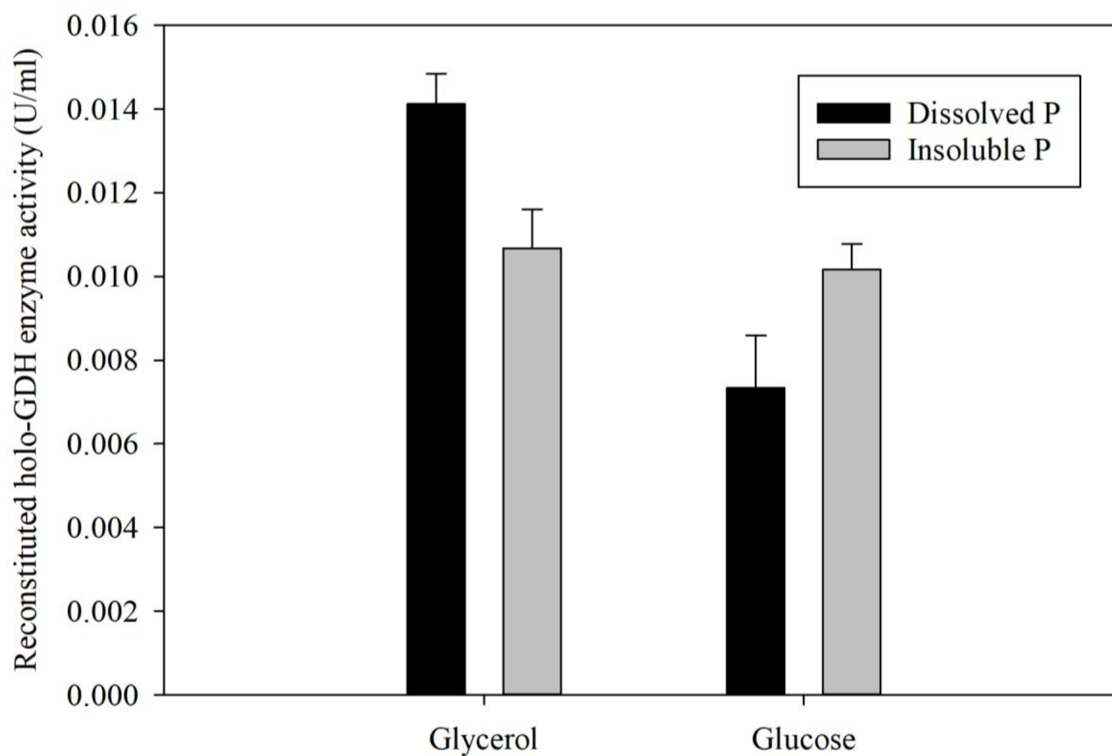

Figure 4-4. Comparison of the activity of reconstituted holo-GDH enzyme with culture supernatant of *B. japonicum* USDA110 from different growth conditions. Error bars represent standard deviation of the mean of three biological replicates. The asterisk indicates the statistical significance ($P < 0.05$) of the mean. The asterisk indicates the statistical significance ($P < 0.05$). Growth condition control: glycerol + dissolved P (KH_2PO_4 and K_2HPO_4), was compared with treatment 1: glycerol + insoluble P $(Ca_3PO_4)_2$, treatment 2: glucose + dissolved P (KH_2PO_4 and K_2HPO_4), and treatment 3: glucose + insoluble P $(Ca_3PO_4)_2$.

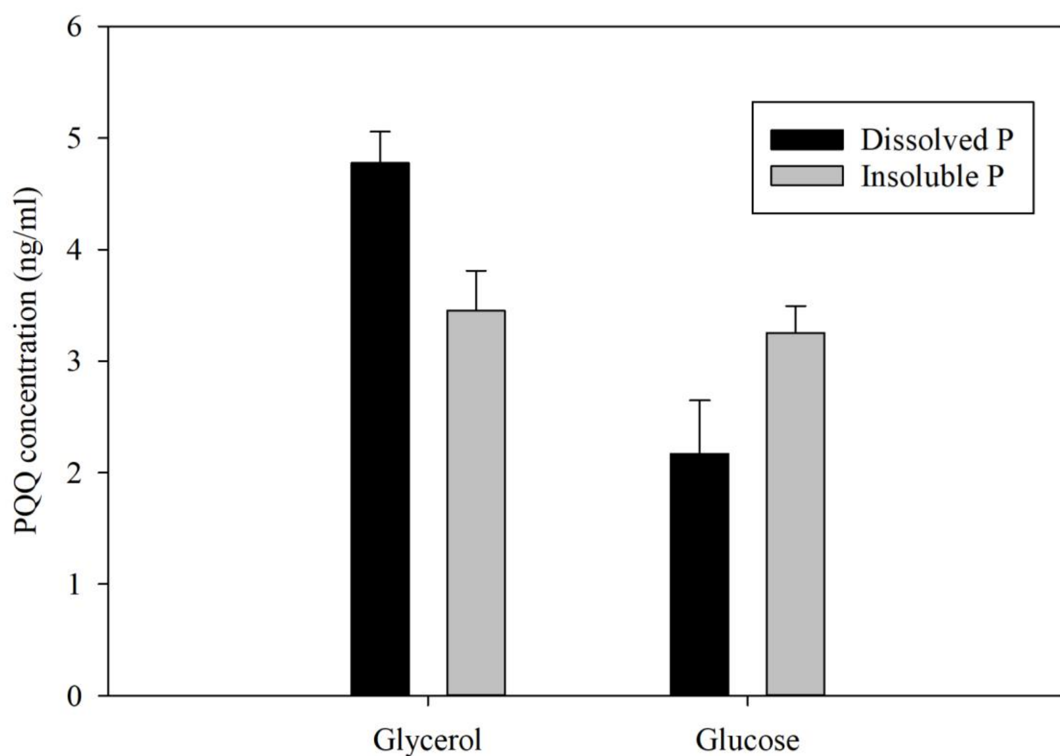

Figure 4-5. Comparison of PQQ concentration in *B. japonicum* USDA110 culture supernatants grown under different conditions. Error bars represent standard deviation of the mean of three biological replicates. The asterisk indicates the statistical significance ($P < 0.05$) of the mean. The asterisk indicates the statistical significance ($P < 0.05$). Growth conditions are control: glycerol + dissolved P (KH_2PO_4 and K_2HPO_4) was compared with treatment 1: glycerol + insoluble P ($(Ca_3PO_4)_2$), treatment 2: glucose + dissolved P (KH_2PO_4 and K_2HPO_4) and treatment 3: glucose + insoluble P($(Ca_3PO_4)_2$).

REFERENCES

Abd-Alla, M. H. 1994. Solubilization of rock phosphates by Rhizobium and Bradyrhizobium. *Folia Microbiol. Off. J. Inst. Microbiol. Acad. Sci. Czech Repub.*, **39**, 53–56.

Ameyama, M., Shinagawa, E., Matsushita, K., & Adachi, O. 1984. Growth Stimulation of Microorganisms by Pyrroloquinoline Quinone. *Growth (Lakeland)*, **48**, 2909–2911.

An, R., & Moe, L. A. 2016. Regulation of pyrroloquinoline quinone-dependent glucose dehydrogenase activity in the model rhizosphere-dwelling bacterium Pseudomonas putida KT2440. *Appl. Environ. Microbiol.*, **82**, 4955–4964.

Antoun, H., Beauchamp, C. J., Goussard, N., Chabot, R., & Lalande, R. 1998. Potential of Rhizobium and Bradyrhizobium species as plant growth promoting rhizobacteria on non-legumes: Effect on radishes (Raphanus sativus L.). *Plant Soil*, **204**, 57–67.

Babalola, O. O., & Glick, B. R. 2012. The use of microbial inoculants in African agriculture: Current practice and future prospects. *J. Food, Agric. Environ.*, **10**, 540–549.

Bernardelli, C. E., Luna, M. F., Galar, M. L., & Boiardi, J. L. 2001. Periplasmic PQQ-dependent glucose oxidation in free-living and symbiotic Rhizobia. *Curr. Microbiol.*, **42**, 310–315.

Boiardi, J. L., Galar, M. L., & Neijssel, O. M. 1996. PQQ-linked extracellular glucose oxidation and chemotaxis towards this cofactor in rhizobia. *FEMS Microbiol. Lett.*, **140**, 179–184.

Chen, P., Toribara, T. Y., & Warner, H. 1956. Microdetermination of Phosphorus. *Anal. Chem.*, **28**, 1756–1758.

Cleton-Jansen, A. M., Goosen, N., Fayet, O., & Van de Putte, P. 1990. Cloning, mapping, and sequencing of the gene encoding Escherichia coli quinoprotein glucose dehydrogenase. *J. Bacteriol.*, **172**, 6308–6315.

Cytryn, E. J., Sangurdekar, D. P., Streeter, J. G., Franck, W. L., Chang, W. S., Stacey, G., Emerich, D. W., Joshi, T., Xu, D., & Sadowsky, M. J. 2007. Transcriptional and physiological responses of Bradyrhizobium japonicum to desiccation-induced stress. *J. Bacteriol.*, **189**, 6751–6762.

Duine, J. , Frank, J., & Zeeland, J. K. V. N. 1979. Glucose Dehydrogenase. *FEBS Lett.*, **108**, 3–6.

Fernández, L. A., Zalba, P., Gómez, M. A., & Sagardoy, M. A. 2005. Bacterias solubilizadoras de fosfato inorgánico aisladas de suelos de la región sojera. *Cienc. Del Suelo*, **23**, 31–37.

Ferri, S., Kojima, K., & Sode, K. 2011. Review of glucose oxidases and glucose dehydrogenases: A bird's eye view of glucose sensing enzymes. *J. Diabetes Sci. Technol.*, **5**, 1068–1076.

Stowers, M.D., & Elkan, G. H. 1984. Gluconate catabolism in cowpea rhizobia: evidence for a ketogluconate pathway. *Arch. Microbiol.*, **137**, 3–9.

Goldstein, A. H. 1995. Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by gram negative bacteria. *Biol. Agric. Hortic.*, **12**, 185–193.

Halder, A. K., & Chakrabartty, P. K. 1993. Solubilization of inorganic phosphate by Rhizobium. *Folia Microbiol. (Praha).*, **38**, 325–330.

Hommes, R. W. J., van Hell, B., Postma, P. W., Neijssel, O. M., & Tempest, D. W. 1985. The functional significance of glucose dehydrogenase in *Klebsiella aerogenes*. *Arch. Microbiol.*, **143**, 163–168.

Isidra-Arellano, M. C., Reyero-Saavedra, M. D. R., Sánchez-Correa, M. D. S., Pingault, L., Sen, S., Joshi, T., Girard, L., Castro-Guerrero, N. A., Mendoza-Cozatl, D. G., Libault, M., & Valdés-López, O. 2018. Phosphate deficiency negatively affects early steps of the symbiosis between common bean and Rhizobia. *Genes (Basel).*, **9**.

Jeon, J.-M., Lee, H.-I., Donati, A. J., So, J.-S., Emerich, D. W., & Chang, W.-S. 2011. Whole-Genome Expression Profiling of *Bradyrhizobium japonicum* in Response to Hydrogen Peroxide. *Mol. Plant-Microbe Interact. MPMI*, **24**, 1472–1481.

Kalayu, G. 2019. Phosphate solubilizing microorganisms: Promising approach as biofertilizers. *Int. J. Agron.*, **2019**.

Kato, C., Kawai, E., Shimizu, N., Mikekado, T., Kimura, F., Miyazawa, T., & Nakagawa, K. 2018. Determination of pyrroloquinoline quinone by enzymatic and LC-MS/MS methods to clarify its levels in foods. *PLoS One*, **13**.

Keele, B. B., Hamilton, P. B., & Elkan, G. H. 1970. Gluconate catabolism in *Rhizobium japonicum. J. Bacteriol.*, **101**, 698–704.

Khairnar, N. P., Misra, H. S., & Apte, S. K. 2003. Pyrroloquinoline-quinone synthesized in *Escherichia coli* by pyrroloquinoline-quinone synthase of *Deinococcus radiodurans* plays a role beyond mineral phosphate solubilization. *Biochem. Biophys. Res. Commun.*, **312**, 303–308.

Khan, A. A., Jilani, G., Akhtar, s. M., Saqlan, M. S., & Rasheed, N. M. 2009. Phosphorus

Solubilizing Bacteria: Occurrence, Mechanisms and their Role in Crop Production. *J. Agric. Biol. Sci.*, **1**, 48–58.

Khan, S. M., Zaidi, A., Ahemad, M., Oves, M., & Wani, A. P. 2010. Plant growth promotion by phosphate solubilizing fungi - Current perspective. *Arch. Agron. Soil Sci.*, **56**, 73–98.

Kousa, S., Labidi, N., Debez, A., & Abdelly, C. 2005. Effect of P on nodule formation and N fixation in bean. *Agron. Sustain. Dev.*, **25**, 389–393.

Kumar, A., Kumar, A., & Patel, H. 2018. Role of Microbes in Phosphorus Availability and Acquisition by Plants. *Int.J.Curr.Microbiol.App.Sci*, **7**, 1344–1347.

López-ortega, M. P., Criollo-campos, P. J., & Gómez-vargas, R. M. 2013. Characterization of diazotrophic phosphate solubilizing bacteria as growth promoters of maize plants. **15(2)**, 115–123.

Love, M. I., Huber, W., & Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2. *Genome Biol.,***15(12)**, 1-21.

Naveed, M. 2016. The Life History of Pyrroloquinoline Quinone (PQQ): A Versatile Molecule with Novel Impacts on Living Systems. *Int. J. Mol. Biol.*, **1**, 29–46.

Puehringer, S., Metlitzky, M., & Schwarzenbacher, R. 2008. The pyrroloquinoline quinone biosynthesis pathway revisited: A structural approach. *BMC Biochem.*, **9**.

Rodríguez, H., & Fraga, R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv.*, **17**, 319–339.

Sharma, B. S., Sayyed, Z. R., Trivedi, H. ., & Gobi, A. T. 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *J. Clin. Microbiol.*, **35**, 3305–3307.

Stowers, M. D. 1985. Carbon metabolism in *Rhizobium* Species. *Metab. Clin. Exp.*, **27**, 89–108.

Taw, M. N., Lee, H. I., Lee, S. H., & Chang, W. S. 2015. Characterization of MocR, a GntR-like transcriptional regulator, in *Bradyrhizobium japonicum*: its impact on motility, biofilm formation, and soybean nodulation. *J. Microbiol.*, **53**, 518–525.

Walpola, charana B., & Yoon, M.-H. 2012. Prospectus of phosphate solubilizing microorganisms and phosphorus availability in agricultural soils: A review. *African J. Microbiol. Res.*, **6**, 6600–6605.

Zhu, F., Qu, L., Hong, X., & Sun, X. 2011. *Isolation and Characterization of a Phosphate-Solubilizing Halophilic Bacterium Kushneria sp. YCWA18 from Daqiao Saltern on the Coast of Yellow Sea of China*. **2011**.

CHAPTER 5

CONCLUSIONS

Symbiotic nitrogen fixation (SNF) by rhizobia in association with legumes is a major component of the N cycling in the biosphere. It takes place in the specialized root organs called nodules where the rhizobia are differentiated into the special metabolic phase called bacteroids and reduce atmospheric N_2 gas into the readily usable form of N (i.e., ammonia) with the aid of nitrogenase under microoxic conditions. Several strains of nitrogen-fixing rhizobia have been identified as potential N biofertilizers and the use of these strains has a huge impact on maintaining soil fertility and improving crop production in agriculture. However, the introduction of these strains into the field has become challenging due to their reduced survivability after applying to seeds or the soil. High soil temperature, soil acidity, nutrient limitation, moisture deficiency, and desiccation have been identified as main stressors for rhizobia in the field which prevent them from surviving in the soil and establishing a symbiotic relationship with their host plants. Additionally, P deficient soils retard growth and development of both plants and microsymbionts. Since, P is an essential nutrient to carry out SNF successfully, there is an emerging requirement of a P biofertilizer to maintain enough dissolved P levels in soil and mitigate adverse effects of synthetic fertilizers. The symbiotic relationship between *B. japonicum* and soybean is the most widely studied symbiotic system and thus *B. japonicum* is well established N biofertilizer. This study was carried out to explore the advanced strategies to enhance the ability of the *B. japonicum* to respond and tolerate major soil stressors, heat stress and acid shock, and to develop a robust inoculum for field applications. Whole genome transcriptomic profiling showed that 49 genes involved in regulatory functions, 23 translation related genes and 44 transport and binding related genes were up-regulated while more

cellular processes associated genes were down-regulated in response to heat stress. This is in accordance with that during the heat stress response the bacterium, tends to suppress the cellular processes such as chemotaxis, movements, cell division and detoxification while inducing the genes encoding for heat shock protein products. Furthermore, nitrogen fixation related genes have been down regulated under heat stress indicating that the heat stress has a negative effect on nitrogen fixation and the symbiotic activities of the bacteria. Responding and overcoming the heat stress minimizing cellular damages and toxic compound assimilation would be challenging situation to the cells under which total cellular mechanisms are focusing only on cell survivability. In such circumstances, all the other cellular and physiological machineries would be shut off to save energy reserves to fight back the consequences of heat shock. Any metabolic pathway that does not crucial for cell viability and longevity would be retarded or totally discontinue until cells are fully recovered. Therefore, heat stress directly impacts the rhizobial ability to carry out successful nitrogen fixation.

Another important finding of the study on related to heat stress is that the genes that were highly upregulated in response to heat shock might not be that essential for the cell survival. This was reflected during the mutagenesis studies performed with two significantly upregulated genes *blr7740* and *blr2203*. Loss of these genes did not affect the growth of *B. japonicum* USDA110 either in normal or heat shock conditions attributes to the compensation of these two gene functions by other genes with the similar functions. This shows that the heat shock response of the bacteria is more complicated and interconnected system that ensures the malfunctioning of one or two genes do not affect the cell's ability to protect itself against the stress conditions. The same fact was proved with the mutagenesis studies done with *blr2694* gene, VirG like two component

response regulator which was upregulated under acid shock. The mutant strain *blr2694* was also recovered growth rates and survival % of the wild type under normal and acid shock conditions.

Specially, heat shock proteins act as universal stress responsive agents in the cell which was evident by induction of *blr7740* under acid shock condition as well. They are not stress stimuli specific, but the intracellular changes commonly associated with any type of environmental stimuli.

Under acid shock, several genes related to multidrug efflux pumps were highly stimulated to pump out excessive protons out from the cells. In this case the importance of that genes was revealed in the mutagenesis studies done with the *blr7593* gene. Even though the mutant *blr7593* could resume the regular growth as the wild type under normal growth conditions it shows drastic reduction of the cell survivability in response to acid shock and that was attributed to its highest fold induction. Therefore, *blr7593* is a vital gene for the survival of *B. japonicum* under acidic soils and its importance is highlighted. This gene could be a potential candidate for genetic engineering advancement of the *B. japonicum* inoculants.

Interestingly, *B. japonicum* was found to solubilize inorganic phosphates which would be beneficial property for rhizobia as a biofertilizer. Apparently, *B. japonicum* has been already established as a N biofertilizer in sustainable agricultural systems and well recognized in terms of its contribution to the global N pool. Given the second most decisive factor in agriculture, P limitation and associated drawbacks of synthetic P fertilizers have drawn much attention on development of a promising novel approach for ecologically bioavailable source of P to crop production. In this study the efficiency of *B. japonicum* in inorganic P solubilization is well demonstrated.

Taken together, after improving its ability to withstand the major field stressors such as heat and acid shocks, *B. japonicum* can be distributed as a dual functioning biofertilizer that can supply both N and P required for crop growth under harsh soil conditions. This will be the green light for a sustainable way of feeding the increasing world hunger.

APPENDIX

Table A- 1. Old and new gene IDs of up and down regulated genes in response to heat shock.

Old Gene ID	New Gene ID (BJA number & ID)
bll5218	BJA_RS26320, ID: 1051477
blr7731	BJA_RS39245, ID: 1047919
bll7025	BJA_RS35595, ID: 1048120
blr4499	Rpal_3144, ID: 6410814
bsr0071	BJA_RS00340, ID: 1049103
blr7740	BJA_RS39290, ID: 1047949
bll4998	bll4998, ID: 1051895
blr0072	BJA_RS00345, ID: 1049076
blr1107	BJA_RS05605, ID: 1048511
blr5221	BJA_RS26335, ID: 1051472
blr7528	BJA_RS38180, ID: 1054911
blr5227	BJA_RS26360, ID: 1051462
bsr1232	BJA_RS06245, ID: 1049478
bll5004	BJA_RS25200, ID: 1051889
blr7979	BJA_RS40505, ID: 1050029
blr0680	BJA_RS03435, ID: 1049300
blr6174	Rvan_2706, ID: 9933510
blr2510	BJA_RS12335, ID: 1050347
bll3015	BJA_RS14940, ID: 1053335
blr1404	BJA_RS07150, ID: 1055149
blr7732	BJA_RS39250, ID: 1047923
bsl4836	bsl4836, ID: 1052144
blr2450	BJA_RS12010, ID: 1050431

bll3190	BJA_RS15835, ID: 1052942
blr2203	BJA_RS10740, ID: 1055345
bll6866	BJA_RS34785, ID: 1049069
bll7938	BJA_RS40305, ID: 1050095
blr0685	BJA_RS03460, ID: 1049266
blr7040	BJA_RS35690, ID: 1048027
bll6865	bll6865, ID: 1049021
bll4354	BJA_RS21685, ID: 1052110
blr4260	BJA_RS21195, ID: 1053272
blr7325	BJA_RS37155, ID: 1054724
bll2253	BJA_RS11005, ID: 1051727
bll6903	BJA_RS34960, ID: 1048649
blr7037	BJA_RS35675, ID: 1048035
blr0639	BJA_RS03230, ID: 1049371
bsr2765	BJA_RS13715, ID: 1054101
bll1804	BJA_RS09050, ID: 1053654
blr1373	BJA_RS06990, ID: 1055118

Table A- 2. Old and new gene IDs of up and down regulated genes in response to acid shock.

Old Gene ID	New Gene ID (BJA number & ID)
blr7593	BJA_RS38510, ID: 1047453
blr2591 (dop)	BJA_RS12785, ID: 1049576
bsl0708 (rpmI)	BJA_RS03580, ID: 1049212
blr7337 (rpoH2)	BJA_RS37220, ID: 1054760
blr5220 (hspE)	BJA_RS26330, ID: 1051473
bll5416 (nusG)	BJA_RS27345, ID: 1047872
bll4012	BJA_RS19900, ID: 1048919
bll0322 (otsA)	BJA_RS01615, ID: 1047884
blr2272	BJA_RS11100, ID: 1051561
bsl4127	BJA_RS20500, ID: 1049723
blr1171 (coxA)	BJA_RS05945, ID: 1049669
bll6866 (fla)	BJA_RS34785, ID: 1049069
bll4906 (nuoL)	BJA_RS24670, ID: 1052053
bll0829 (dnaN)	BJA_RS04185, ID: 1050625
blr7593	BJA_RS38510, ID: 1047453
blr2591 (dop)	BJA_RS12785, ID: 1049576
bsl0708 (rpmI)	BJA_RS03580, ID: 1049212
blr7337 (rpoH2)	BJA_RS37220, ID: 1054760
blr5220 (hspE)	BJA_RS26330, ID: 1051473
bll5416 (nusG)	BJA_RS27345, ID: 1047872
bll4012	BJA_RS19900, ID: 1048919
bll0322 (otsA)	BJA_RS01615, ID: 1047884
blr2272	BJA_RS11100, ID: 1051561
bsl4127	BJA_RS20500, ID: 1049723
blr1171 (coxA)	BJA_RS05945, ID: 1049669
bll6866 (fla)	BJA_RS34785, ID: 1049069

bll4906 (nuoL)	BJA_RS24670, ID: 1052053
bll0829 (dnaN)	BJA_RS04185, ID: 1050625
bsr4726	bsr4726, ID: 1052323
blr7593	BJA_RS38510, ID: 1047453
bll5859	BJA_RS29655, ID: 1050041
bsr4236	BJA_RS21070, ID: 1053512
bsl4014	bsl4014, ID: 1048920
bll6622	BJA_RS33550, ID: 1050322
blr2591	BJA_RS12785, ID: 1049576
blr4827	blr4827, ID: 1052156
bll1466	BJA_RS07505, ID: 1055216
bsl4437	bsl4437, ID: 1052741
bsl7903	BJA_RS40125, ID: 1050252
blr7528	BJA_RS38180, ID: 1054911
bsl2206	BJA_RS10760, ID: 1055343
bll0729	BJA_RS03690, ID: 1049155
bll0333	BJA_RS01670, ID: 1049919
blr2272	BJA_RS11100, ID: 1051561
blr3208	BJA_RS15925, ID: 1052922
bsl6587	BJA_RS33365, ID: 1051508
bll6858	BJA_RS34745, ID: 1052734
bll4784	BJA_RS24030, ID: 1052240
bsr1505	bsr1505, ID: 1055255
blr7827	BJA_RS39725, ID: 1049001
bsl4127	BJA_RS20500, ID: 1049723
blr3200	BJA_RS15885, ID: 1052931
bll5076	BJA_RS25585, ID: 1051796
bll6853	BJA_RS34720, ID: 1049134

blr4701	BJA_RS23600, ID: 1052354
bll0912	BJA_RS04620, ID: 1047871
blr2269	BJA_RS11085, ID: 1051718

Table A- 3. Old and new gene IDs of up and down regulated genes in response to 10 μM PQQ.

Old Gene ID	New Gene ID (BJA number & ID)
blr7475	BJA_RS37920, ID: 1047280
blr4100	blr4100, ID: 1049914
bll2752	BJA_RS13650, ID: 1054117
blr1469	BJA_RS07520, ID: 1055219
bll7642	Dfer_0512, ID: 8224079
blr4223	BJA_RS21000, ID: 1053773
blr3169	BJA_RS15730, ID: 1052965
bsr2400	BJA_RS11760, ID: 1050575
bll6467	bll6467, ID: 1047425
blr1496	BJA_RS07650, ID: 1055246
bll6993	BJA_RS35420, ID: 1047475
blr4936	BJA_RS24835, ID: 1051998
blr5214	blr5214, ID: 1051481
bll1465	BJA_RS07500, ID: 1055215
blr6074	BJA_RS30740, ID: 1051671
blr7345	BJA_RS37260, ID: 1054781
blr0497	BJA_RS02505, ID: 1054212
blr7037	BJA_RS35675, ID: 1048035
bll3998	BJA_RS19830, ID: 1049054
blr1289	BJA_RS06550, ID: 1049326
bll6069	BJA_RS30715, ID: 1051855
bsl7992	BJA_RS40570, ID: 1049952
blr4114	BJA_RS20425, ID: 1049745
bsr6066	BJA_RS30700, ID: 1051856
bll5655	BJA_RS28620, ID: 1053817
blr2611	BJA_RS12910, ID: 1054071

bll7551	BJA_RS38300, ID: 1047323