CONTROL OF IRON REGULATION AND UPTAKE BY THE EXPR/SIN QUORUM SENSING SYSTEM IN *SINORHIZOBIUM MELILOTI*

by

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Dedicated to my parents Dr. A. Raghu Kumar and A. Nagamani without whose support and motivation this would have never been possible.

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DISSERTATION

Presented to the Faculty of

The University of Texas at Dallas

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY IN

MOLECULAR AND CELL BIOLOGY

THE UNIVERSITY OF TEXAS AT DALLAS

May 2020

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Juan González, for his constant encouragement and guidance throughout my graduate study. I discovered the joys of being a scientist from his relentless pursuit to seek knowledge. His enthusiasm and motivation helped me through some of the most challenging phases of my research project. I sincerely thank my committee members, Dr. Spiro, Dr. Palmer and Dr. Burr, for their patience, inputs and guidance throughout my graduate career. A big thank you to my friends from Dr. Reitzer's and Dr. Spiro's lab. This journey has been very memorable because of their company and willingness to help each other.

I want to thank my husband, Harin Pagidimunthala, without whose support this would not have been possible. He has been a pillar of my strength. I would also like to thank my first mentors, my uncle and aunt, D.V.S.Prasaka Rao and D.V.S. Surekha Rao, for instilling a sense of genuine passion and confidence in my growing childhood years. My dear friends Divya, Karthik, Ramya and Harshitha who always believed in me more than I believed in myself. I will always be grateful to them for being a great support system. I also express gratitude to my extended family members, my father-in-law P. Babu Rao, mother-in-law P. Vasantha Kumari, my sister-in-law P. Shirisha for their support. I would also to like to extend my gratefulness to my aunt, Mrs. Kamakshi and my grandmother for their positive influence on my life. Finally, I want to thank my dear son, Adhvik Pagidimunthala, for teaching me to be more patient, to always smile no matter what and never give up in what we believe.

December 2019

CONTROL OF IRON REGULATION AND UPTAKE BY THE EXPR/SIN QUORUM SENSING SYSTEM IN *SINORHIZOBIUM MELILOTI*

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Sinorhizobium meliloti is a gram-negative soil bacterium that establishes a symbiotic association with the legume host *Medicago sativa*. The bacteria use the ExpR/Sin QS system, a cell-cell based communication mechanism, to invade the root nodules and fix nitrogen for the plant. The process of establishing symbiosis between legume and the bacteria requires an interplay of many factors; one of the most pivotal is iron. Studies in the past have shown that legumes involved in symbiosis have a greater requirement for iron and limiting the availability of iron has a tremendous impact on the efficiency of nodulation and nitrogen fixation. This is due to the fact that many of the key enzymes and proteins involved in symbiotic nitrogen fixation, such as nitrogenase use iron as a cofactor, and as a result there is a high demand for iron by the nitrogen-fixing bacteroids in the root nodules. Though iron is one of the most abundant transition metals on the Earth's crust, at a physiological pH, it is both poorly soluble and unavailable. Therefore, microbes have adopted several strategies to obtain iron; one of the most efficient is the use of siderophores, diffusible molecules that are secreted under strict iron-limited conditions with a very high-affinity for the ferric (Fe^{+3}) form of iron.

S. meliloti produces rhizobactin 1021 as its predominant siderophore. The biosynthesis of rhizobactin 1021 is mediated by six genes arranged in the operon *rhbABCDEF*. Additionally, *rhtX* and r*htA*; the genes that code for an outer membrane receptor and a permease, respectively, help in the recognition and transport of the iron-siderophore complexes across the membrane using the energy generated from the ExbBD-TonB complex. As in the case with other siderophores, the synthesis and release of rhizobactin 1021 is positively regulated by *rhrA* and negatively in the presence of iron by the rhizobial iron uptake regulator RirA.

Quantitative Real Time-PCR analyses conducted in our laboratory showed differential expression of the genes involved in the synthesis, transport, and regulation of rhizobactin 1021 in a wild-type strain compared to the QS mutants that lacked either the *sinI* or the *expR* component of the QS system. Symbiosis studies conducted on plants inoculated with a QS capable strain vs. plants inoculated with a QS mutant showed that a wild-type is far more efficient in invading root nodules under iron-limiting conditions. These results suggested that the presence of an intact ExpR/Sin QS system might help *S. meliloti* to cope with iron scarcity. Therefore, in this current study, we set out to understand the possible role of the ExpR/Sin QS in siderophore synthesis and regulation and its influence on plant root nodulation.

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CHAPTER 1

BROAD PERSPECTIVES ON THE IMPORTANCE OF IRON

1.1 Introduction

Iron is an essential element that plays a role in a wide array of biochemical, cellular, and physiological pathways in life. It forms an integral part of iron associated cofactors, the Fe-Sulfur clusters found in metalloproteins, cytochrome complexes, enzyme machinery like catalases, hydrogenases, nitrogenases, and in heme [1]. In cellular processes iron is necessary for DNA replication, the electron transport chain during respiration, and the protection of cells against oxidative stress. Iron, one of the most abundant transition metals on the Earth's surface, exists in two forms: Ferrous (II) and Ferric (III). Before the oxygenation of the Earth's atmosphere, Fe (II) could be accessible to living cells due to its relative solubility at neutral pH in aqueous solutions [1]. However, the emergence of photosynthesis resulted in the release of molecular oxygen into the atmosphere, changing drastically the reactive capabilities of iron [1]. The predominant form of iron switched from the relatively soluble ferrous state (with saturating concentration of 0.1 M at neutral pH) to the highly insoluble ferric form (with saturating concentration of 10^{-18} M at neutral pH), thereby reducing the concentration of available iron to three-fold below the optimal levels required for basic cellular metabolism [1]. Furthermore, ferrous iron is extremely reactive in the presence of oxygen, generating toxic reactive oxygen species (ROS) that can destroy cellular proteins and DNA [1]. Therefore, iron is both poorly available and extremely toxic in the presence of oxygen [1]. It is indeed challenging for most living organisms to obtain iron from their environment, as well as to maintain a balance between the amounts of iron uptake from the surroundings vs. the amount of free iron in the cell to prevent iron associated toxicity by ROS.

1.2 Distribution of iron and iron-bound proteins in animals

In animals, iron is an important component of heme (oxygen transport), myoglobin (a heme-containing protein that carries and stores oxygen in muscle cells), storage proteins like ferritins, iron-sulfur clusters, and other iron-containing centers of many redox enzymes that are involved in the mitochondrial respiratory chain, and DNA synthesis (ribonucleotide reductase) [2]. The average amount of iron in higher-order animals like mammals is 3-5 g, the majority of which (60-65%) is present in red blood cells (RBCs) in the form of hemoglobin, and the rest is stored in intracellular iron storage molecules called ferritins that comprise 30% of the total iron pool [3]. Ferritins are a class of iron storage proteins that can hold up to 4500 atoms of iron per molecule [4]. Apart from heme and ferritins, transferrin is another widely found iron associated protein in the circulating blood of animals. Transferrin can bind up to two molecules of Fe (III) and plays an important role in quenching the iron atoms released by the lysis of old or dead RBCs and circulating them back to ferritins [5]. As a result, any free iron released into the bloodstream is strictly recycled to maintain iron homeostasis, restricting the free serum iron concentration to less than 10-24 M/L [3]. Therefore, homeostasis not only plays a critical role in maintaining iron levels but also protects the cell from iron-related toxicity [25].

1.3 Importance of iron in plant growth and metabolism

Iron is one of the most limiting micronutrients for plant growth and metabolism, mostly due to its low solubility in aerobic environments [6]. In plants, iron is essential for chlorophyll synthesis and is involved in the maintenance of chloroplast structure and function [7]. Typically, 80%-90% of the iron in plants is found in the photosynthetic cells, where it is crucial for the biosynthesis of cytochromes, the electron transport system, the synthesis of iron-sulfur clusters, nitrogen fixation,

and DNA synthesis [7]. It is also necessary for plant hormone synthesis such as ethylene, abscisic acid, or lipoxygenase (compounds that are active in many plant developmental pathways and their adaptive responses to changing environmental conditions) [7]. Being the fourth most abundant element in the lithosphere, iron is generally present in high quantities in soils; however, its bioavailability is severely limited at neutral and high pH (especially for plants growing in alkaline soils) [6]. Iron limitation affects almost one-third of the cultivable land on earth, representing a major concern for agriculture [8]. Lack of iron causes the decline of photosynthetic components such as ferredoxin (Fd), an iron-sulfur protein involved in the chloroplast oxidoreductive pathway [8]. Symptoms of iron deficiency in plants involve chlorosis, poor root formation, growth retardation and, death [9]. Chlorosis has been mostly attributed to the inhibition of chlorophyll synthesis, which requires iron-containing enzymes [10]. Therefore, plants have evolved two such adaptive mechanisms to take up solubilized iron from scarcely available sources: Strategy I (reduction strategy) by non-graminaceous and flowering plants (dicotyledons) and Strategy II (the chelation strategy) by graminaceous plants [7]. Non-graminaceous and flowering plants employ a three-component system: i) a cell-membrane bound iron-deficiency-induced ferric reductase at the root surface, ii) H^+ extrusion that promotes the reduction of Fe^{+3} to Fe^{+2}, and iii) in certain cases the release of reducing and/or chelating substances by the roots [7]. Once iron is solubilized, Fe^{+3} is reduced to Fe^{+2} by a membrane-bound Fe^{+3} reductase oxidase, and the iron (II) is transported into the root by an iron-regulated transporter (IRT1) [7]. Figure 1 shows two distinct strategies for iron uptake in plants. The second iron-uptake system is the one employed by graminaceous plants (grasses), which is characterized by a two-component system that involves an iron deficiency-induced release of specific Fe^{+3}-chelating compounds called phytosiderophores and a high-affinity

transport system in the root cells for the uptake of Fe^{+3} bound phytosiderophores [11]. Phytosiderophores (PS) are low molecular-weight non-proteinaceous amino-acids released by graminaceous species such as barley, wheat, maize, and sorghum under iron deficiency and are of great agricultural significance for the acquisition of iron [12]. Besides iron, phytosiderophores can mobilize zinc, manganese, and copper [13]. Despite this non-specific mobilization, PS are able to acquire appreciable amounts of iron in iron-deficient soils and are of significance for chlorosis resistance in graminaceous species [11]. As a result, graminaceous species exhibiting these two components are highly efficient at acquiring iron.

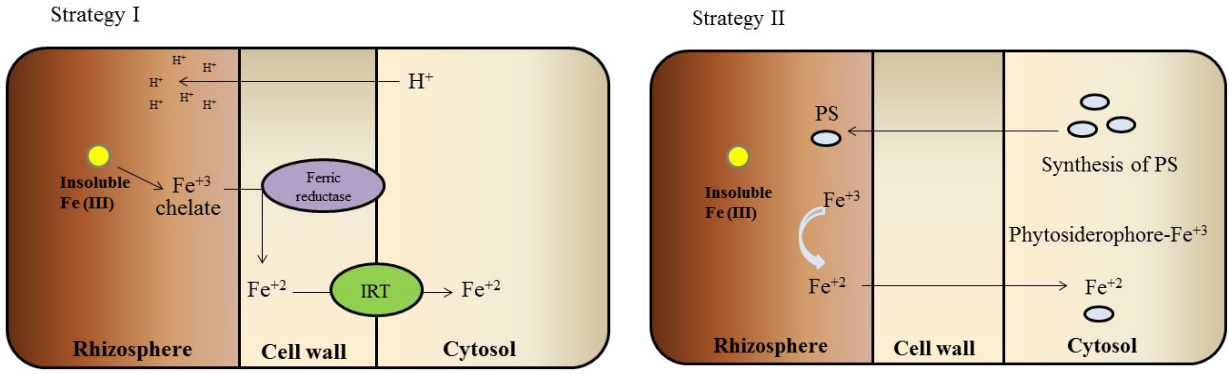

Figure 1. Iron uptake mechanisms in plants. (a) Ferric form of iron is reduced to ferrous form by a cell membrane-bound ferric reduction oxidase (FRO), followed by uptake by the iron-regulated transporter IRT1. (b) Synthesis of iron-chelating phytosiderophores (PS) and secretion followed by re-uptake of chelated iron (III) [15].

1.4 Iron transport and storage in plants

Most of the iron that enters the plant via the roots needs to be transported to tissues where high iron-dependent enzyme activity resides, such as leaves and seeds [14]. This is accomplished with the help of metal ion transporters, iron chelators and long-distance transport systems [14]. However, due to its

toxicity and low solubility, iron is generally complexed with chelators for translocation to other plant tissues without causing damaging redox reactions [14].

Physiological and molecular studies have identified some principal chelators inside the plant body, such as citrate, nicotinamide (NA), and mugineic acid (MA, a phytosiderophore) (14,16). The tri-Fe (III) tri-citrate complex is responsible for the translocation of iron into the xylem (a type of vascular tissue in plants involved in long-distance transport of water and nutrients from roots to stems, and leaves) ([14], [9]). NA, a non-protein amino acid that chelates to iron, is synthesized from *S*-adenosyl methionine by nicotinamide synthase (NAS) [14]. NA is a precursor of mugineic acid, and chelates zinc and other divalent cations in addition to iron [14]. An important sink tissue for iron is the leaves, which are active sites for photosynthesis [14]. Here, iron (III) enters to the symplast (inner side of the plant cell membrane) and is reduced to iron (II) by ferric reduction oxidases (FROs) and is found as Fe^{+2}-NA [14]. A large portion of this reduced iron (II) chelated to NA is transported to plastids and mitochondria for the biosynthesis of heme and iron-sulfur clusters. Finally, iron is remobilized from leaves to other sink tissues via the phloem (a plant tissue involved in transporting soluble organic compounds made during photosynthesis) [14]. The terminal destination of iron is often considered to be the seed, where iron stores are important for germination before the seedling has developed root and takes up nutrients from the soil [14]. Figure 2 shows the distribution of iron across different sink tissues in plants.

Plants have two major storage mechanisms for iron: 1) sequestration into vacuoles and 2) plant ferritins [14]. Vacuoles of plant cells are multifunctional organelles that play a central role in plant development. They function as reservoirs for iron and other metal ions in germinating seeds prior to uptake from the external environment [17]. The vacuolar iron transporter VIT1 was

first identified in *Arabidopsis thaliana*, and it plays a major role in the localization of iron in the seed [14]. In *vit1* mutants, the iron content of embryos was found to be similar to the wild type, but iron was no longer localized in the vacuoles of roots, suggesting that *vit1* is essential for iron localization in seeds [18].

Ferritins, on the other hand, are important iron storage proteins present ubiquitously in all biological kingdoms [19]. The portion of total iron stored in ferritin in the seeds varies significantly among different species, with approximately 60% in peas (legumes) to less than 5% in *Arabidopsis* seeds [14]. Plants ferritins are typically located in plastids, which are double-membraned sac-like organelles involved in synthesis and storage of food, with exceptions like cereal grains where most iron is present in vacuoles [14].

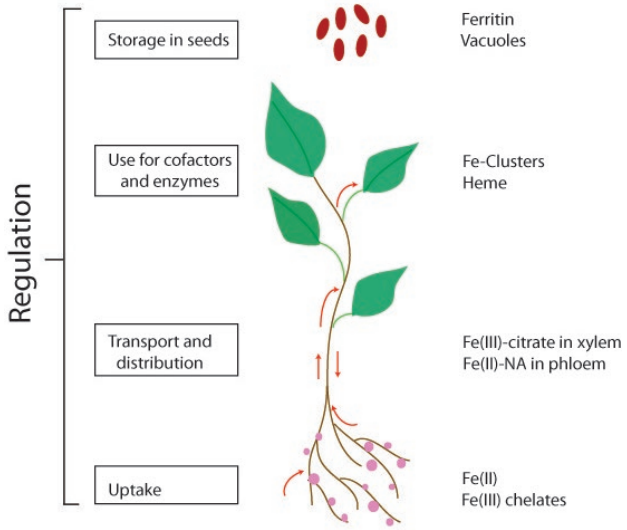

Figure 2. Uptake and distribution of iron across various sink tissues [14]. Adapted by permission from the Royal Society of Chemistry (Connorton JM, Balk J, Rodriguez-Celma J), ©2017 RSC. All rights reserved.

1.5 Microbial iron uptake

Rhizobia

The rhizobia (soil bacteria) can live as free-living bacteria or in symbiosis with leguminous plants. The success of these organisms in each milieu largely depends on their ability to sense the environment to assess nutrient availability and optimize cellular systems for nutritional acquisition [1]. Rhizobia belong to a diverse family of alpha-proteobacteria, classified by their ability to establish a symbiotic relationship with plants in which the bacteria actively fix atmospheric nitrogen to ammonia [20]. Some examples of nitrogen-fixing rhizobia are *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Bradyrhizobium* species that live in soil and may enter a symbiosis with leguminous plants such as peas, alfalfa, clover and soybean [21]. Free-living and symbiotic nitrogen-fixing rhizobia have several mechanisms to take up iron and compete for scarce iron in the soil. These include the release of high-affinity iron chelators or scavengers that can bind to iron in the environment and transport it back to the cells (such as siderophores) and heme acquisition systems that use heme as a source of iron [1]. Free-living rhizobia employ several mechanisms to obtain iron, such as the release of *de novo* siderophores, or they steal siderophores produced by other bacteria [1]. In addition to utilizing heme as a source of iron, bacteria may also reduce the ferric form of iron to the ferrous iron by the action of ferric reductases [22]. Therefore, it is not surprising that a considerable amount of variation exists in the range of iron sources utilized by different rhizobia.

Pathogenic bacteria

Pathogenic bacteria that infect eukaryotic hosts are posed with a different set of challenges for acquiring iron. For instance, in high order animals like mammals, iron is bound to proteins like

transferrins and lactoferrins (a protein of the transferrin family present in secretory fluids such as milk, saliva, tears, and nasal secretions) [1]. Similarly, heme is bound to hemoglobin, and other cellular proteins like haemopexin (a plasma protein expressed in the liver with a high binding affinity to heme) and haptoglobins (proteins produced by the liver to sequester free hemoglobin from circulation), limiting the availability of free iron for the bacteria. Pathogens often use low iron as a signal for the induction of virulence genes [23]. For example, the Shiga-like toxin I of enterohaemorrhagic bacteria *Escherichia coli* is induced by iron starvation [24]. Pathogens counter this iron restriction imposed by their hosts through the use of siderophores and/or by acquiring iron directly from host iron-bound proteins via receptor-mediated transport systems specific for host-iron complexes [24]. However, the main drawback of receptor-mediated transport systems is that the microbe must be able to synthesize specific receptors for each iron source. Therefore, bacteria have evolved to compete successfully for iron. Two such widely studied iron uptake mechanisms in bacteria are described below: a) hemophores and b) siderophores. Hemophores are proteins with a high affinity for heme [26]. These molecules can specifically recognize heme-associated proteins and acquire iron. However, the role of hemophores is restricted to only heme iron resources, making them very inefficient under conditions of low or no heme [1]. To compensate for this, bacteria have developed an efficient strategy that is capable of exploiting iron from any source, thus making it one of the most widespread and successful mechanisms of iron sequestration [1]. This is based on a shuttle mechanism that uses small diffusible high-affinity iron chelators called siderophores.

1.6 Siderophore based iron acquisition

Siderophores are low molecular weight (<1000 Da), high-affinity iron chelators secreted by a wide spectrum of microorganisms [4]. Siderophores are secreted under conditions of very low iron availability, i.e., at a concentration of 1 μM or lower [3]. The association constant of siderophores with iron is in the order of 10^{50}, making them the strongest known iron chelators [3]. Siderophores are synthesized in the cytoplasm and secreted across the cell membrane in response to iron limitation [1]. The iron (III) bound siderophores are recognized by outer membrane receptors OMRs present on the cell surface [27]. With the help of metal transporters, these complexes are moved across the cell membrane and released into the cytoplasm. Any bacteria in the surrounding environment (not only siderophore producing bacteria) with cognate outer membrane receptors can recognize and transport these iron-siderophore complexes; therefore siderophores are referred to as Public Goods or Collective Traits [28]. The ability to take up siderophores is particularly advantageous for opportunistic pathogens that infect humans, as co-infecting bacterial cells benefit from these shared traits [4]. Many bacteria produce or are capable of utilizing siderophores produced by other organisms [4]. Any mutations that negatively affect the synthesis or transport of siderophores have been shown to decrease the fitness of pathogenic bacteria in causing infections [30]. For example, studies on chemically derived mutants of *Corneybacterium diphtheriae* showed a very high expression of diphtheria toxin in iron-replete media which is otherwise repressed in a wild-type strain (29, 31). When these mutants were grown in low iron media, they were found to be defective in factors required for iron uptake and exhibited severe growth defects [31]. This is due to the fact that a wild-type strain of *C. diphtheriae* under iron-limiting conditions produces corynebactin, a siderophore, whereas a corynebactin-deficient mutant

is defective in both growth and iron transport [32]. However, the growth of these mutants could be rescued by the addition of culture supernatants containing *C. diphtheriae* siderophore suggesting that defects in corneybactin synthesis or transport could play a vital role in the fitness of this pathogenic bacteria [31].

1.7 Types of siderophores

The basic structure of a siderophore consists of a hexadentate octahedral complex formed with Fe (III) in such a way that there are three bidentate ligands [33]. This arrangement minimizes theverall change in entropy caused when the ligand binds to ferric iron [34]. Siderophores were thus originally classified based on the chemical nature of the ligands that chelate Fe (III) into types: catecholates (also phenolates) and hydroxamtes [33]. Some of the well-studied examples of siderophores are enterobactin (produced by *Escherichia coli* and *Salmonella typhimurium*), a catecholate, and ferrichrome produced by fungi, a hydroxamate. The isolation of rhizobactin produced by *Rhizobium meliloti* DM4 marked the discovery of a new class of novel siderophores (which do not share any similarity to catecholates or hydroxamates) that use ethylenediamine as the ligand [35]. Additionally, *Rhizobium meliloti* 1021 produces a citrate-based dihydroxamate siderophore referred to as rhizobactin 1021 that differs from rhizobactin in structure [43]. Figure 3 shows some of the different classes of siderophores.

1.8 Mechanisms of siderophore synthesis

The mechanisms for the synthesis of siderophores rely predominantly on the chemical nature of the siderophore groups. However, the pathways are stratified into two types based on the enzymes

that catalyze the biosynthetic pathway: non-ribosomal peptide synthetase dependent (NRPS) and NRPS independent. NRPS is a large family of modular multi-enzyme complexes

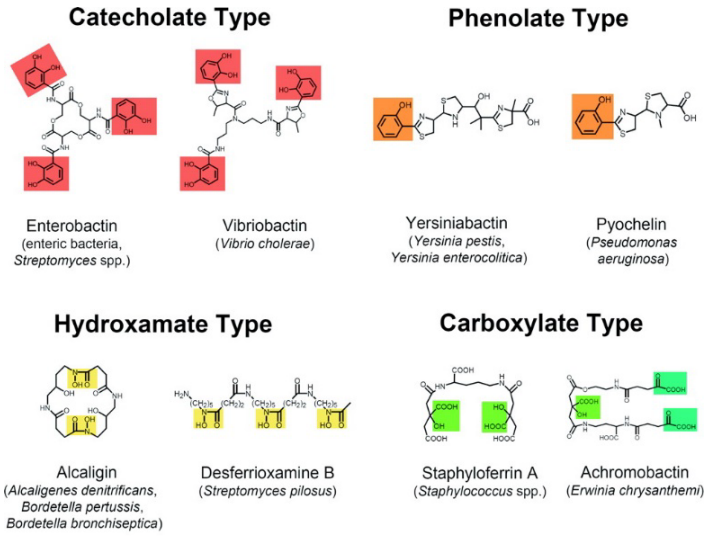

Figure 3. Examples of several different siderophores and the organisms that produce them. The ligands involved in binding to iron are highlighted as follows: catecholates are in red, phenolates in orange, hydroxamates in yellow, citrate deriving is in green, carboxylates in blue-green. Adapted by permission from Microbiology and Molecular Biology Reviews (Marcus Miethke, Mohamed A. Marahiel, 2007), ©2007 American Society for Microbiology. All rights reserved.

that recruit and assemble the molecular machinery required for siderophore biosynthesis [4, 36]. NRPSs are responsible for the synthesis of aryl-capped siderophores, and it is through this pathway that most bacteria synthesize their secondary metabolites [4]. Some of the well-studied siderophores in this category are enterobactin (*Escherichia coli*), vibriobactin (*Vibrio cholerae*), pyochelin, and pyoverdine (*Pseudomonas aeruginosa*). In the NRPS independent pathway, a diverse spectrum of enzymes namely monooxygenases, aminotransferases, decarboxylases, and aldolases, are recruited individually depending on the type of siderophore synthesized [4]. This

can be best seen in the hydroxamates and carboxylate siderophores like aerobactin (produced by enteric bacteria), and staphylobactin (in *Staphylococcus aureus*) [36].

1.9 Siderophore mediated iron transport in gram-negative bacteria

Most gram-negative bacteria are equipped with OMRs on their cell surface that recognize and bind to iron-siderophore complexes with high specificity [27]. These OMRs, in general, exhibit variability and many bacteria are found to have multiple OMRs each providing the bacterium with specificity for various siderophores. The synthesis of these receptors is normally not initiated under iron-rich conditions, but it is induced by iron starvation [1]. This is a strategy to prevent the entry of phages, bacterial toxins, and antibiotics that target OMRs as a potential route of entry into a cell [1]. OMRs have very high substrate specificity and require energy to actively transport iron-siderophore complexes against a concentration gradient [37]. The energy for this transport across the membrane is provided by a complex of integral and transmembrane proteins located in the cell membrane called the TonB−ExbB−ExbD complex. Once the iron-siderophore complexes are recognized by OMRs and released into the periplasm, they are shuttled by periplasmic binding proteins (PBPs) to the cognate permeases In this complex, ExbBD code for integral cell membrane proteins, and TonB is associated with both the inner and the outer membrane, with a large part of the protein occupying the periplasmic present on the surface of the inner membrane [39]. The ATP-binding cassette (ABC) transporters such as permeases, a sub-domain of ABC superfamily of transporters, have a periplasmic binding protein and an inner membrane complex that is energized with an ATPase [40]. With the help of ABC metal transporters, the iron-loaded siderophores are released into the cytosol [41].

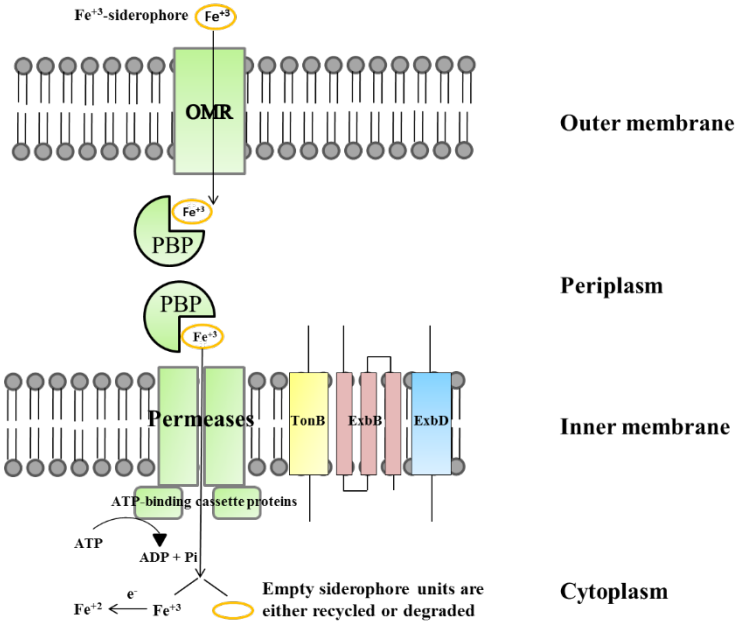

Figure 4. Schematic representation of iron uptake via siderophores in gram-negative organisms. Siderophore mediated iron acquisition in gram-negative bacteria requires an OMR, a periplasmic binding protein (PBP), and an inner membrane ABC transporter (usually a permease). Transport through the OMR requires the action of the TonB-ExbBD complex to provide energy in the form of proton motive force. Adapted with permission from Biochimica et Biophysica Acta (BBA) - Biomembranes (Karla D. Krewulak, Hans J. Vogel), ©2008 Elsevier. All rights reserved.

As periplasmic proteins encounter a variety of substrates during the shuttling of proteins, they exhibit less substrate specificity when compared to OMRs [1]. In the cytoplasm, the Fe (III) in the iron-siderophore complex is reduced to the Fe (II) form by the action of ferric reductases, thereby releasing Fe (II), which is then readily incorporated into the appropriate proteins [1]. The empty siderophore units are either degraded or exocytosed for re-use [1]. A schematic of TonB-ExbBD mediated iron uptake in gram-negative bacteria is shown in Figure 4.

1.10 Transport across the periplasm and cytoplasmic membrane in rhizobia In gram-negative rhizobia, several variations of the periplasmic binding proteins and inner membrane transporters have been observed [41]. For example, in *Rhizobium leguminosarum*, the *fhu* genes

of the *fhuABCD* operon (*fhuA* codes for an OMR, *fhuD* a periplasmic transporter and *fhuBC* an inner membrane ATPase) are induced under iron-limiting conditions, and these genes are involved in the uptake and transport of vicibactin (the siderophore produced by *R. leguminosarum*) [1]. A similar mechanism is seen in *Bradyrhizobium japonicum* for heme uptake where *hmuR* codes for an OMR, *hmuT*, a periplasmic transporter, and *hmuUV* an inner membrane ATPase [42, 50]. However, the siderophore uptake system in *Sinorhizobium meliloti* is different from *R. leguminosarum* and *B. japonicum*. The genes for the synthesis of rhizobactin 1021 are encoded by the rhizobactin 1021 operon *rhbABCDEF* located upstream of *rhtA*, which is the gene that codes for the OMR [43]. *rhrA* (located between *rhbABCDEF* and *rhtA*) is a positive regulator of synthesis and transport of rhizobactin 1021 [43]. RhrA belongs to AraC-like family of transcriptional regulators (commonly known as AFTRs) that constitute one of the largest groups of regulatory proteins in bacteria and are involved in a variety of cellular processes including carbon metabolism, stress response, and virulence [44]. Figure 5 shows the genes involved in the synthesis, transport, and regulation of rhizobactin 1021.

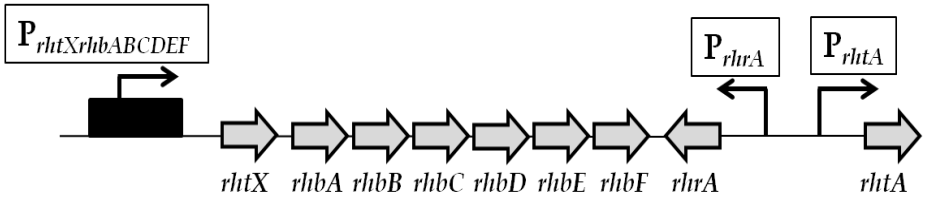

Figure 5. The rhizobactin 1021 operon. The synthesis, transport, and regulation of rhizobactin 1021 is controlled by a total of nine genes. *rhbABCDEF* code for proteins involved in the assembly and synthesis of rhizobactin 1021; *rhtX,* a permease, *rhtA* is an OMR, and *rhrA*-an Ara-C like regulator of the rhizobactin regulon. Three promoters control the genes of the rhizobactin operon. The promoters are labeled as P followed by the genes regulated. Adapted with permission from The Journal of Bacteriology (Lynch D, O'Brien J, Welch T, Clarke P, Cuív PO, Crosa JH, O'Connell M), ©2001, American Society for Microbiology. All rights reserved [43].

1.11 Regulation of iron acquisition in rhizobia

Genes involved in iron uptake are tightly controlled by various regulators in the cell. Fur (ferric uptake regulator), deemed as a global regulator ubiquitous to gram-negative bacteria, was first described in 1978 as a repressor of iron-regulated genes in *E. coli* and *Salmonella typhi* [21]. However, research in the last decade has identified several novel iron regulators such as RirA and Irr that controls iron uptake in different gram-negative bacteria [21]. The iron regulator RirA was first described in *R. leguminosarum* by Todd *et al.* [45]. RirA belongs to a family of Rrf2 putative transcription regulators [46]. Some examples of this family are Rrf2 from *Desulfovibrio vulgaris*; IcR, a repressor of genes coding for Fe-S cluster assembly proteins in *E. coli*; and NsrR of *Nitrosomonas europaea*, a nitrate sensitive transcriptional repressor [21]. These helix-turn-helix proteins have highly conserved cysteine residues and can act as both repressors and activators [47]. The iron-responsive regulator Irr which belongs to the Fur family of regulators is mostly confined to the members of alpha-proteobacteria, with homologs of Irr present in most rhizobia as well as some close rhizobia relatives such as the *Brucella* [21]. First reported in *B. japonicum*, Irr controls iron transport and represses heme synthesis under iron limiting conditions (Figure 6) [42].

1.12 *Bradyrhizobium japonicum*

Irr was originally identified in *Bradyrhizobium japonicum* by Hamza *et al*. in 1998 [48]. Close homologs of Irr have been found in *Nitrobacter hamburgensis* and *Bradyrhizobium* sp BTAi1 [48]. Unlike Fur, Irr does not respond to Fe availability in the cell. It instead forms a complex with the heme biosynthetic enzyme (ferrochelatase) and responds to the status of heme at the site of synthesis [49]. Irr regulates the biosynthesis of δ–aminolevulinic acid dehydratase, which catalyzes the second step in the heme biosynthesis pathway [21]. Irr-mediated regulation is critical

in ensuring that protoporphyrin production, a precursor of heme, does not exceed iron availability [21]. Iron-replete conditions allow heme synthesis by ferrochelatase, which results in Irr binding to ferrochelatase and consequent inactivation of Irr [21]. However, when iron levels are low, protoporphyrin accumulates, enabling the dissociation of Irr from ferrochelatase and ultimately repressing heme biosynthesis [21]. Thus, the production of protoporphyrin is controlled to ensure that it does not exceed iron availability [21].

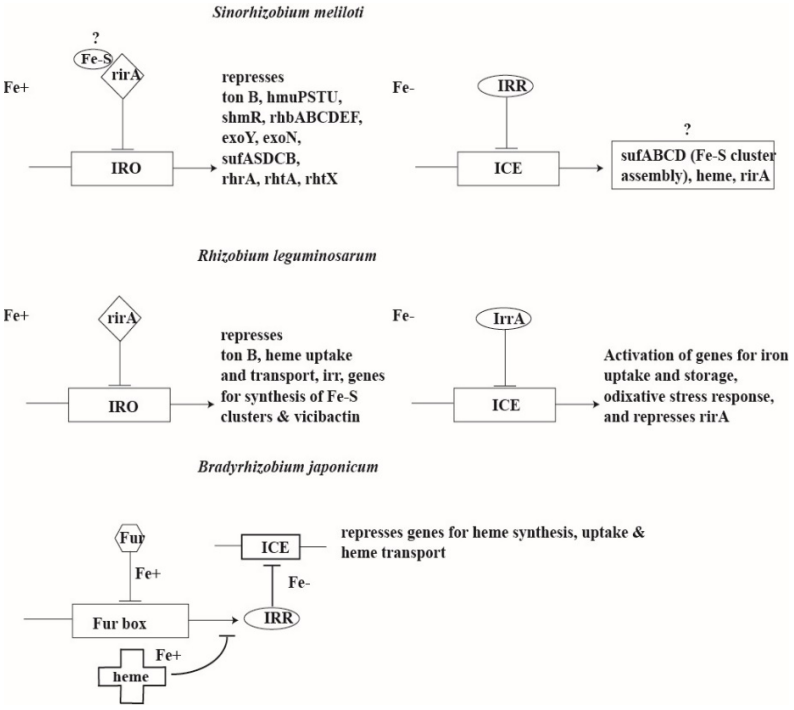

Figure 6. Comparative models of iron-regulatory mechanisms in select rhizobia. RirA, rhizobial iron regulator; Irr, iron response regulator, Fur; ferric uptake regulator. IRO (iron response operator), ICE (iron control element) and Fur box are the predicted DNA binding sites for RirA and Irr. Question marks refer to target genes that are not yet well studied. Adapted with permission from FEMS Microbiology Reviews (Rudolph G, Hennecke H, 2006), ©Oxford University Press. All rights reserved.

1.13 *Rhizobium leguminosarum*

R. leguminosarum produces vicibactin as its primary siderophore. The synthesis (*vbs* genes) and

uptake (*fhu* genes) of vicibactin, as well as some of the ABC metal transporter genes, are repressed in response to iron by a transcriptional regulator termed RirA [45]. Proteomic studies by Todd *et al.* confirmed that RirA is a global regulator of more than 80 proteins [45]. The *cis*-acting regulatory sequences of many genes repressed by RirA share a common conserved sequence called the iron response element (IRO), which is the predicted DNA binding site of RirA [21]. In addition to RirA, *R. leguminosarum* also has Irr, which represses heme synthesis and uptake when iron is abundant [48, 51]. Mutants of *irr* in *R. leguminosarum* showed deregulation of heme biosynthesis, although the precise genes were not identified [48]. Therefore, RirA is under dual control, being subjected to auto-regulation under high-Fe conditions, and Irr-dependent-repression under low-Fe conditions [51].

1.14 *Sinorhizobium meliloti*

RirA regulated iron-responsive genes in *Sinorhizobium meliloti* were first identified using a whole-genome microarray study by Chao *et al.* in 2005 [47]. More than 40 genes involved in heme acquisition, and iron transport were found to be induced in a *rirA* mutant when compared to the wild-type, suggesting that these genes are normally repressed by RirA [21]. *S. meliloti* produces rhizobactin 1021 as its primary siderophore. The biosynthesis of rhizobactin 1021 is mediated by eight genes that are involved in the regulation, synthesis, and the transport of rhizobactin 1021 [43]. RhtA codes for an outer membrane receptor that recognizes rhizobactin 1021 and brings it into the periplasm using energy generated from the TonB complex, whereas *rhtX* codes for a permease [43]. As in the case with other siderophores, the synthesis and release of rhizobactin 1021 is regulated by the intracellular concentration of iron [43]. *rhrA*, a positive regulator is involved in the upregulation of *rhbABCDEF*, *rhtA*, and *rhtX* genes required for siderophore

biosynthesis, uptake, and transport under conditions of strict iron limitation [43]. However, the mechanisms of iron acquisition, uptake, and regulation under iron limiting conditions in *S. meliloti* have not been very well characterized thus far. Figure 7 shows the regulation and transport of rhizobactin 1021 in *S. meliloti*.

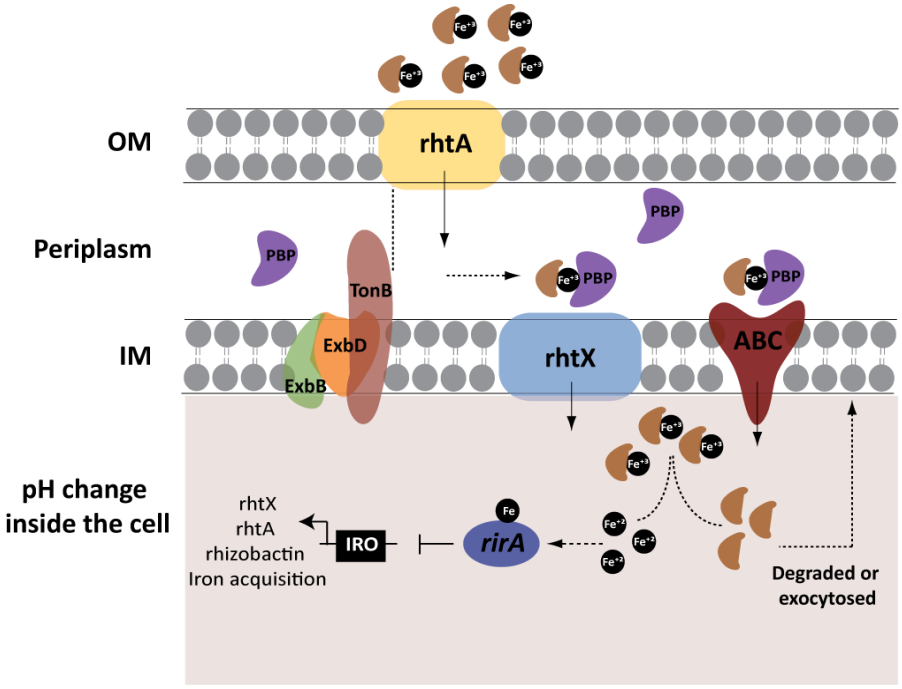

Figure 7. Regulation and transport of rhizobactin 1021 in *S. meliloti*.

CHAPTER 2

ROLE OF IRON IN SYMBIOTIC NITROGEN FIXATION

2.1 Introduction

Nitrogen is the most common growth-limiting factor for plants in terrestrial ecosystems [52]. Although dinitrogen is the most abundant element in the atmosphere, it is biochemically unavailable for plants and most microbes [52]. There are two main natural processes for the fixation of atmospheric nitrogen: lightning and biological nitrogen fixation. Lightning contributes to only ~1% of ammonia of the net nitrogen fixed per year. Biological nitrogen fixation is carried out by microorganisms and contributes almost 60% of the total nitrogen fixed per year, while 30% is fixed by industrial processes [53]. Nitrogenase, the key enzyme essential for nitrogen fixation, consists of two multi-subunit metalloproteins: component I, a dinitrogenase which has the active site for nitrogen reduction, and component II, a dinitrogenase reductase which couples ATP hydrolysis to electron transfer [22]. Component I and II of the nitrogenase enzyme complex form a heterodimer using Fe-Mo as cofactors and constitute up to 10-12% of the total protein in a nitrogen-fixing bacterium.

The symbiotic relationship between nitrogen-fixing rhizobia and the legume host plant is a result of an intercellular signaling pathway between the host and the symbiont [20]. The result of this symbiosis is to form root nodules, within which the bacteria convert atmospheric nitrogen to ammonia that can be used by the plant [54]. Bacteria and the host plant use chemical signals to regulate genes involved in nodulation [55]. Briefly, plant root hairs secrete phenolic compounds called flavonoids that attract the bacteria towards the roots and trigger the expression of the nodulation genes resulting in the release of Nod factors [55]. Nod factors are composed of a

backbone of *N*-acetylglucosamine residues, a fatty acyl moiety with variable length and different degrees of saturation, and various side chains on the backbone, all of which are signal-specific [20]. Once the Nod factors are recognized by the plant, the rhizobia invade the plant roots resulting in root hair curling and the formation of infection threads [55]. Inside the root nodule, the bacteria differentiate to form morphologically distinct structures called bacteroids that express the genes necessary for nitrogen fixation [55].

The interior of the root nodule provides a favorable environment for nitrogen fixation via rhizobia by limiting the amount of oxygen, as the nitrogenase enzyme is extremely sensitive to oxygen, which irreversibly deactivates the enzyme [22]. Leghemoglobin, a plant-produced heme associated protein found in the nitrogen-fixing root nodules of legumes, acts as an oxygen carrier and maintains an oxygen concentration that is low enough for the nitrogenase enzyme to function [22]. An estimated 25% of soluble iron within the nitrogen-fixing root nodule is present in leghemoglobin, suggesting that iron plays an important role in maintaining the nodule environment [56]. Other molecules that have a very high demand for iron during nitrogen fixation are cytochromes (hemoproteins known for their role in redox reactions during electron transport chain), hydrogenases (metallo-enzymes that use Fe-Fe or Ni-Fe to catalyze the reversible oxidation of molecular hydrogen for redox reactions) and ferredoxins (iron-sulfur proteins that mediate electron transfer in a range of metabolic reactions) [22]. For the synthesis of these iron-containing compounds, bacteria must acquire an adequate supply of iron. It has been shown that legumes involved in symbiotic nitrogen-fixation have a higher requirement for iron [57]. Deficiency of iron acquisition from the environment or unavailability of iron in the soil can have a considerable impact on the interaction between legume host and rhizobia [58]. Studies by Gill *et al*. and O'Hara

et al. have identified inefficiency in nodulation, and delayed onset of nitrogen fixation in plants such as chickpea, peanut, lentil, and soybean under conditions of iron limitation, indicating that iron is an important requirement in the differentiation of rhizobia to bacteroids in the host [59]. At nodule maturity, approximately 44% of the iron within soybeans has been shown to be present in the nodule compared to 31% in leaves, 7% in the seeds, and ~5% in roots [43].

Under conditions of iron limitation, free-living rhizobia express TonB-dependent receptors induced by the iron response regulator however, this is not the case with bacteriods that have established symbiosis in a host plant (60, 22). Evidence for this includes the down regulation of siderophore uptake system, Ton-B dependent receptors and ABC metal transporters in bacteriods (61, 22). Mutations in ABC metal transporters and the energy transducing Ton-Exb system have no effect on the function of established symbiosis, suggesting that once differentiated in the host, bacteriods do not obtain iron via the high affinity transport system [43]. This could be due to the slightly acidic pH of the root xylem, where Ferrous (II) is the predominant form of iron and siderophores are not required for its uptake [22]. Besides, nodules may also take up Ferrous (II) iron directly as reports of ferric chelate reductases; enzymes that reduce the Ferric form (III) iron to Ferrous (II) are present on the surface of roots [62]. However, *S. meliloti* was shown to have increased nodule occupancy under iron limiting conditions, compared to mutant strains with impaired siderophore uptake system [63]. Therefore, the ability to use iron chelators might provide an additional advantage to the bacteria and have an impact on the effectiveness of the resulting symbiosis.

2.2 Role of the ExpR/Sin quorum sensing in symbiotic nitrogen fixation

Quorum sensing (QS) was first discovered in *Vibrio fischeri,* a marine bacterium that produces

light when cultures reach the late-logarithmic phase of the growth [64]. Studies by Hastings, Nealson, and others led to the understanding of this phenomenon, in which population density-dependent gene regulation controls luminescence via signaling molecules called autoinducers [65]. Though initially referred to as autoinduction, it was later renamed quorum sensing (QS) in a review written by Fuqua *et al.* in 1994 [66]. QS regulates a variety of processes, such as the formation of biofilms, motility, chemotaxis, symbiotic nitrogen fixation, siderophore biosynthesis, production of virulence factors, exopolysaccharides and antibiotic synthesis (67, 74, 75).

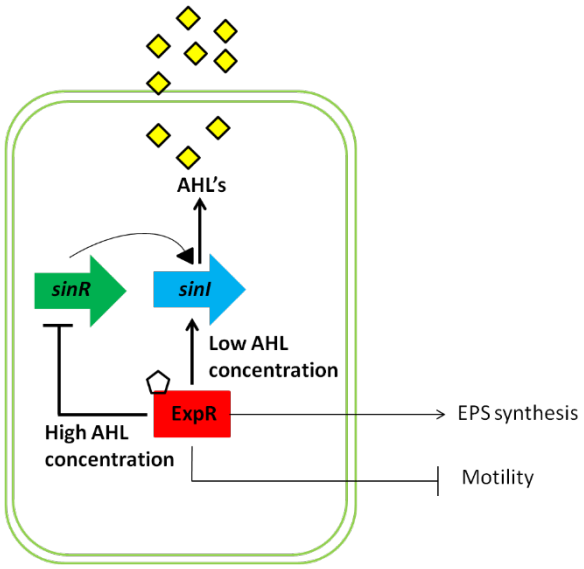

Figure 8. The ExpR/Sin quorum-sensing system in *S. meliloti*. The transcription of *sinI*, which encodes the AHL synthase, is induced by SinR [72]. *sinI* transcription can also be induced by the AHLs that bind to ExpR at low AHL concentrations; however, at high AHL's concentration, *sinR* is repressed by the AHLs- ExpR complex [57]. Furthermore, the AHL-ExpR complex also induces the expression of genes related to EPS synthesis and represses the expresses of genes for motility [73].

One of the best-studied quorum-sensing systems in symbiotic nitrogen fixation can be seen in *S. meliloti*, a gram-negative soil bacterium of the *Rhizobiaceae* family that can establish symbiosis with *Medicago sativa* [55]. The QS system in *S. meliloti* consists of an autoinducer

synthase which produces *N*-acyl homoserine lactones (AHLs), and a transcriptional regulator that binds to the AHLs in a concentration-dependent manner to influence the transcription of target genes. *S. meliloti* has two quorum-sensing systems: the ExpR/Sin system and the Tra system [55]. The Tra system is seen in select strains of *S. meliloti* and is known to mediate plasmid transfer at high population densities [68]. The Sin system in *S. meliloti* is composed of two proteins: SinI, which is responsible for the synthesis of the quorum sensing signaling molecules, *N*-acyl homoserine lactones, and SinR a transcriptional regulator responsible for the expression of *sinI* [69]. A second transcriptional response regulator, ExpR, works in conjunction with the AHLs to regulate a variety of genes involved in nodulation and nitrogen fixation [20]. ExpR is known to either mediate a positive feedback loop by inducing the expression of *sinI* or a negative feedback regulation by down-regulating the expression of *sinR,* depending on the concentration of AHLs [70]. Genome-wide microarray analysis by Hoang et al., 2009 on cultures of *S. meliloti* showed that at low-AHL concentrations, *expR* activates genes for motility (*visN*, *visR*, and *rem*) [55]. However, as the concentration of AHLs increases with the increase in cell population density, motility genes are repressed, suggesting a diverse role for the ExpR regulator [70]. Figure 8 is a schematic of the ExpR/ Sin quorum-sensing system in *S. meliloti*.

2.3 Concluding Remarks

S. meliloti is a gram-negative soil bacterium that can establish a symbiotic association with legumes of the following genera: *Medicago*, *Trigonella*, *and Melilotus* [20]. These bacteria use QS to invade the root nodules and fix nitrogen for the plant, while the plant provides nutrients to the bacteria. The process of establishing symbiosis between the legume and the bacteria requires an interplay of many factors, one of which being iron [58]. Previous studies have shown that

legumes involved in symbiosis have a higher requirement for iron and that limiting the availability of iron has a tremendous impact on the efficiency of nodulation and nitrogen fixation [57]. This is due to the fact that many of the key enzymes and proteins involved in symbiotic nitrogen fixation use iron as a cofactor, and the nitrogen-fixing bacteriods in root nodules require iron for cellular metabolism, DNA replication, electron transport chain, and protection against oxidative stress [71]. Though iron is one of the most abundant transition metals on Earth's crust, at physiological pH, iron is poorly soluble and unavailable (10^{-18} M) [1]. It is, therefore, challenging for microbes to obtain iron from the environment, and this is circumvented by the production of siderophores [5].

Genome-wide microarray analysis by Hoang *et al.* showed differential expression of several genes involved in iron acquisition, iron-sulfur cluster synthesis, siderophore biosynthesis, and transport in strains of *S. meliloti* that have an intact QS system *vs.* QS mutants, suggesting a potential role of QS in siderophore mediated iron uptake [55]. Preliminary experiments conducted in our laboratory on plants inoculated with a QS capable strain *vs*. plants inoculated with a QS mutant grown under iron limiting conditions indicate that, a wild type strain is more efficient in invading the root nodules, suggesting that the presence of a functional QS system might aid *S. meliloti* to cope with iron stress. In addition, plant symbiosis studies on seedlings of *Medicago sativa* inoculated with strains of *S. meliloti* showed increased nodule occupancy under iron limiting conditions, compared to mutant strains with impaired siderophore uptake systems [63]. Therefore, the ability of rhizobia to take up iron chelated to siderophores appears to provide a competitive advantage and may impact the effectiveness of the resulting symbiosis. In this study, we set out to

understand the role of QS in siderophore mediated iron uptake and examine its effect on nodulation efficiency and symbiotic nitrogen fixation.

2.4 Aims and Objectives

The main objective of this study is to understand the role of QS in siderophore mediated iron uptake and the impact on nodulation and symbiotic nitrogen fixation. To get a holistic view of the effect of QS on rhizobial iron regulation, the three aims listed below were systematically studied.

Aim I- To investigate the role of quorum sensing in siderophore mediated iron uptake.

Aim II- To quantify the expression of genes involved in the biosynthesis and transport of rhizobactin 1021 in *S. meliloti* using quantitative real time-PCR.

Aim III- To examine the effects of QS-mediated siderophore production on nodulation efficiency and symbiotic nitrogen fixation in *Medicago sativa*.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strains and growth media.

The bacterial strains and plasmids used in this study are listed in Table 1. *Sinorhizobium meliloti* strains were grown at 30° C, 250 RPM in LB (Luria-Bertani) media supplemented with 2.5 mM $MgSO_4$, and 2.5 mM $CaCl_2$ (referred to as LB/MC) [57]. *Escherichia coli* cultures were grown in LB media with the appropriate antibiotics at 37° C, 250 RPM. For RNA extraction, *S. meliloti* cultures were grown in low-phosphate minimal glutamate mannitol (MGM) media, as described here [75]. For iron-restricted growth we used the low-iron tryptone- yeast (TY) extract media with added 2, 2'-bipyridyl at a concentration of 200 µM [47]. Cultures were grown in plastic 250 mL vented culture flasks and incubated at 30° C, 250 RPM, and harvested at an OD_{600} of 0.8-1.0. For siderophore assays, *S. meliloti* cultures were grown in Vincent minimal media (VMM) with no added iron for iron-deficient growth [72]. A final $FeCl_3$ concentration of 0.37 µM was used for iron-minimal, 37 µM for iron-sufficient and 60 µM for iron-replete growth in VMM media. When appropriate, antibiotics were added to the growth media at the following concentrations: streptomycin (Sm) 500 µg/ml, neomycin (Nm) 200 µg/ml, trimethoprim (Tp) 200 µg/ml, gentamicin (Gm) 100 µg/ml, and kanamycin (Km) 25 µg/ml. All glassware used in the preparation of low-iron and Chrome Azurol S media was washed with 6 M HCL and thoroughly rinsed with distilled water.

3.2 DNA manipulations

Construction of rhizobactin 1021 mutants.

Internal fragments of *rhbA, rhbB, rhbC, rhbD, rhbE, rhbF, rhrA, rhtA,* and *rhtX* were cloned into pKmobΩ19HMB, creating recombinant vectors harbored in *E. coli* S17-λ*pir* (Table 1). These vectors were provided by Dr. Anke Becker from the Phillips University of Marburg, Germany. Vectors were transferred via bi-parental mating into *S. meliloti* Rm1021, and recombinants were selected by plating on minimal media with the respective antibiotics. Mutants were confirmed by PCR and phage ΦM12 was used to transduce the mutation in Rm8530 wild-type and Rm8530 *sinI* strain [74]. The primers for constructing the rhizobactin 1021 mutants are listed in Table 2.

Construction of rhizobactin 1021 mutants.

The *S. meliloti* Rm1021 *rirA* strain was kindly provided by Dr. Hisayuki Mitsui, Graduate School of Life Sciences, Tohoku University, Japan. The *rirA* mutation was confirmed by PCR and transduced using the phage ΦM12 into Rm8530 *sinI*, and Rm8530 wild-type to create *rirA* mutants with and without an intact quorum-sensing system (Table 2).

3.3 CAS siderophore assay.

The CAS medium for the detection of siderophore production was prepared by the method described by Schwyn and Neilands 1987, with the modifications described by Reigh and O'Connell [35]. Supernatants of *S. meliloti* cultures grown in VMM containing various concentrations of iron chloride were mixed in a 1:1 ratio with the CAS assay solution [47]. Once

equilibrium was reached, the absorbance was measured at OD_{630} nm. The higher the absorbance, the lower the siderophore activity.

3.4 RNA isolation and cDNA synthesis.

Bacterial cultures were grown for 24 hours in LB media with magnesium and calcium and appropriate antibiotics. A 1:100 dilution was used to inoculate 20 mL of TY media with added 200 μM of 2, 2- bipyridyl when iron-depleted growth was required. Cultures were grown at 30° C, 250 RPM and harvested at an OD_{600} 0.8-1.0 by centrifugation (14,500 rpm for 2 minutes at 4° C), immediately flash-frozen in liquid nitrogen, and then stored at -80° C for future use. RNA isolation was performed using the RNeasy Mini Kit (Qiagen) with minor modifications. Briefly, cells were thawed on ice and suspended in 10 mM Tris HCL (pH 8.0) and RLT buffer provided with the Qiagen kit (with added β-mercaptoethanol). Cells were then transferred to FastProtein tubes (Qbiogene) and disrupted using an MP FastPrep-24 ribolyser (40 seconds, speed 6.5). Spin columns were used to purify according to the instruction manual provided with the RNeasy Mini Kit. Two steps of RNase-free DNase treatments were performed on the samples: 1) Qiagen on-column RNase-free DNase and 2) Ambion TURBO RNase-free DNase, followed by RNA clean up. RNA samples were eluted, and the concentrations were determined by Nanodrop. DNA contamination was assessed by qRT-PCR. cDNA was synthesized using the Ambion RETROscript kit according to the manufacturer's protocol. One microgram of total RNA was used per cDNA synthesis reaction.

Table 1. Strains and Plasmids

Strains or plasmids	Relevant characteristics	Reference or source
Sinorhizobium meliloti		
Rm8530 wild-type	Su47 *str-21*, *expR*+, Sm^R	[80]
Rm8530 *rhrA*	*rhrA*::Nm	This work
Rm8530 *rirA*	*rirA*::Gm	This work
Rm8530 *rhtA*	*rhtA*::Nm	This work
Rm8530 *rhtX*	*rhtX*::Nm	This work
Rm8530 *rhbA*	*rhbA*::Nm	This work
Rm8530 *sinI*	*sinI*::Tp	[81]
Rm8530 *sinI rhrA*	*sinI*::Tp *rhrA*::Nm	This work
Rm8530 *sinI rirA*	*sinI*::Tp *rirA*::Gm	This work
Rm8530 *sinI rhtA*	*sinI*::Tp *rhtA*::Nm	This work
Rm8530 *sinI rhtX*	*sinI*::Tp *rhtX*::Nm	This work
Rm8530 *sinI rhbA*	*sinI*::Tp *rhbA*::Nm	This work
Escherichia coli		
Dh5a	See source	Life Technologies
Plasmids		
pK19mobΩHMB	Insertion vector	Anke Becker

3.5 Quantitative real-time PCR.

Oligonucleotide sequences used for qRT-PCR are listed in Table 3. The master mix for qRT-PCR analysis contained 0.3 µM of sense primer, 0.3 µM of antisense primer, 0.5X of SYBR

green, 0.5 Omni Mix HS PCR beads (1.5 U *Taq* DNA polymerase, 10 mM Tris-HCL [ph 9.0], 50 mM KCL, 1.5 mM $MgCl_2$, 200 µM deoxynucleotide triphosphate, and stabilizers), and 1 µl of cDNA sample in a total reaction volume of 25 µl. qRT-PCR was performed using Cepheid Smart Cycler, version 2.0, as previously described [82]. Expression analyses were done in three independent biological replicates. The expression of *SMc00128* was used an internal control and for normalization, as previously described (55).

Table 2. Primers for Mutant Construction.

Gene	Forward Primer	Reverse Primer
rhbA	ATTTCTTTCCCTACCCCTATGC	GATTACCAACGGGGGCAAGA
rhbB	CTTCTGTTTTCCCGCCATCG	GCGGATTGAGCAGGGTGAGC
rhbC	GCTATGGCAGGATTTCTCGC	GGGTTGGTGATTTGAAGGTAGAC
rhbD	TAGAAGACATCGCAGCCTATA	CGGTAGCAGAACATCAGTTTGGC
rhbE	CTTCACCCGCTTCCTCTTTC	ACCGAACTTCTGGAACGAT
rhbF	CTTCACCCGCTTCCTCTTTC	ACCGAACTTCTGGAACGAT
rhtX	TCGGATCCTATCATGACAAT	ACCGTTCCAGCATCCAGACT
rhtA	CTGGAAGAAATCGTAGTCAC	GTTATGGATACCGAAATT
rhrA	TCTATTTCCCCAGAACAATG	GCCTTCCGCCAGTATGAGAC
rirA	GACGAAGCAAACCAACTA	CAGGAGGAAGTTGATCTG

3.6 Plant symbiosis assays.

Infection assays of *Medicago sativa* were performed to determine the nodule invasion efficiency of the *S. meliloti* strains listed in Table 1. Five-milliliter *S. meliloti* cultures were grown in LB broth with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ with the appropriate antibiotics at 30° C for 48

hours. Cultures were washed five times with sterile water, and 1 ml of a 1:1000 dilution was used to inoculate germinated seedlings of *M. sativa* and grown on Jensen's agar plates with and without added 1% $FeCl_3$ as previously described [84]. Plants were grown in a 16-hour light cycle at 20° C and 65% humidity. Plant roots were inspected weekly, and routine plant health measurements were performed beginning the second-week post-inoculation. Nitrogen-fixing nodules, empty nodules, and plant height (and any identifiable leaf characteristics) were recorded for approximately 75 plants per strain per media condition. Data shown were collected fourth-week post-inoculation.

Table 3. Primers for qRT-PCR

Gene	Forward Primer	Reverse Primer
rhbA	CGAAATCTTCAACGGAGTT	GAGGCAATCGAGATAGGATC
rhbB	CGACGCTTTGAAGATTCTCA	GAGACGTAACGGAAGAGAAC
rhbC	GAGCCAAGCGAGTCCGTCTT	TGCCGAGAACCGAATTGATGAGG
rhbD	TCGTGCCGCAATGGAAGATC	ACATCGTCCTTCGCCCAGTA
rhbE	CAGGAATACGATCACTATTG	ATATTACGGCTTCGATATTG
rhbF	CACACTTCATCAAAGAACTC	AAGGCAAGATAATCGGAATA
rhtX	CAGCATCCAGACTGACAATC	CAGCATCCAGACTGACAATC
rhtA	TACTATGGAATTGGCAACTAC	GATGAGCGATTGAGATTGAT
rhrA	CAGGATAGTGAACGGAAACG	GTTCGCAAGGTTCTGTTCTA
rirA	CGTTTCCGAACTGTTCCTTT	AACTGTCTTCCGTCACCTTC

CHAPTER 4

RESULTS

4.1 Genome-wide expression studies suggest that the ExpR/Sin quorum-sensing system regulates iron-responsive genes in *S. meliloti*.

Microarray analyses of *S. meliloti* were conducted in our laboratory on wild type and QS mutants to identify genes controlled by the ExpR/Sin QS system at different phases of the bacterial growth cycle (21). From these experiments, several putative iron-responsive genes, metal transporters, siderophore synthesis, and transport genes were found to be differentially regulated in a QS mutant (Rm8530 *sinI* and Rm1021) compared to the wild-type (Rm8530) strain in an ExpR/Sin dependent manner. As the cell population-density reached the mid-log phase of the growth cycle, we observed the activation of several genes involved in the synthesis and transport of rhizobactin 1021 in a wild-type strain of *S. meliloti*. Additionally, in the absence of an intact QS system as seen in the mutants Rm8530 *sinI* and Rm1021 *expR*, the genes for siderophore biosynthesis and uptake were significantly reduced (Table 4). These preliminary results suggested that the ExpR/Sin QS system might play a role as an activator of the siderophore biosynthesis and transport genes. Moreover, previous studies conducted in our laboratory identified over 200 genes as being QS controlled at the mid-log phase of the bacterial growth cycle and one of them was *rhrA*, a transcriptional activator of the rhizobactin 1021 operon that was found to be differentially expressed in wild-type *vs.* the QS mutant strains (31). Therefore, we set out to examine the role of the ExpR/Sin QS system in the regulation of rhizobactin 1021, first by detecting the actual siderophore activity using

a liquid CAS assay followed by studying the genes involved in the synthesis and transport of rhizobactin 1021 by qRT-PCR.

Table 4. Candidate Genes from Microarray Data (30, 21)

		Fold change at indicated phase*					
		early log		mid-log		late log	
gene	gene function	*sinI vs. wt*	*expR vs. wt*	*sinI vs. wt*	*expR vs. wt*	*sinI vs. wt*	*expR vs. wt*
rhbC	rhizobactin synthesis	16.01	4.06	-1.16	-4.36	-1.2	-3.41
rhbD	rhizobactin synthesis	4.56	1.36	-1.46	-4.17	-1.13	-2.41
rhbE	rhizobactin synthesis	4.37	1.29	-1.23	-4.28	-1.02	-3.5
rhbF	rhizobactin synthesis	5.66	1.29	-1.27	-6.51	-1.06	-2.64
rhtA	rhizobactin receptor	9.28	2.02	-1.12	-2.92	-1.06	-1.66

Values indicate a change in gene expression in the wild-type (*wt*) strain compared to that in QS mutants (*sinI* and *expR*). *Bacterial cultures were collected at OD_{600} of 0.2 (early log phase), 0.8 (mid-log phase) and 1.2 (late-log phase). Negative values indicate the down-regulation of genes (21).

4.2 The ExpR/Sin QS system in *S. meliloti* regulates iron uptake via siderophores biosynthesis.

To detect siderophore production, we used the liquid CAS assay, which is based on an iron-dye complex that changes color upon the removal of iron by strong chelators such as a siderophore (32). Wild-type and QS mutant strains of *S. meliloti* were grown in vincent minimal media (VMM) with various iron concentrations: iron-deplete media (no source of added iron also referred to as 0 μM), iron-minimal media (0.37 μM Fe), iron-sufficient media (37 μM Fe), and iron-replete (60

μM Fe) media and the cell supernatants were mixed in liquid CAS solution in a 1:1 ratio. If the bacteria produce siderophores under iron-limiting conditions, the Fe (III) from the dye complex will be removed resulting in a reduction in the absorbance of the solution (Figure 9).

Results from the liquid CAS assay showed that under conditions of strict iron-limitation, a wild-type strain has 3-to 4-fold higher siderophore activity when compared to a QS mutant (Figure 10). We assayed the supernatants of cultures grown in iron-replete media (37 μM of $FeCl_3$ or higher) and observed no siderophore production. Sterile VMM also showed no change in absorption (Figure 11). The CAS assay results indicated that a wild-type strain has higher siderophore activity when compared to a QS mutant under iron-limiting conditions, suggesting a possible role of the ExpR/Sin QS system in siderophore synthesis. We next used qRT-PCR to determine if QS played a role in the expression of siderophore biosynthesis and transport genes in *S. meliloti*.

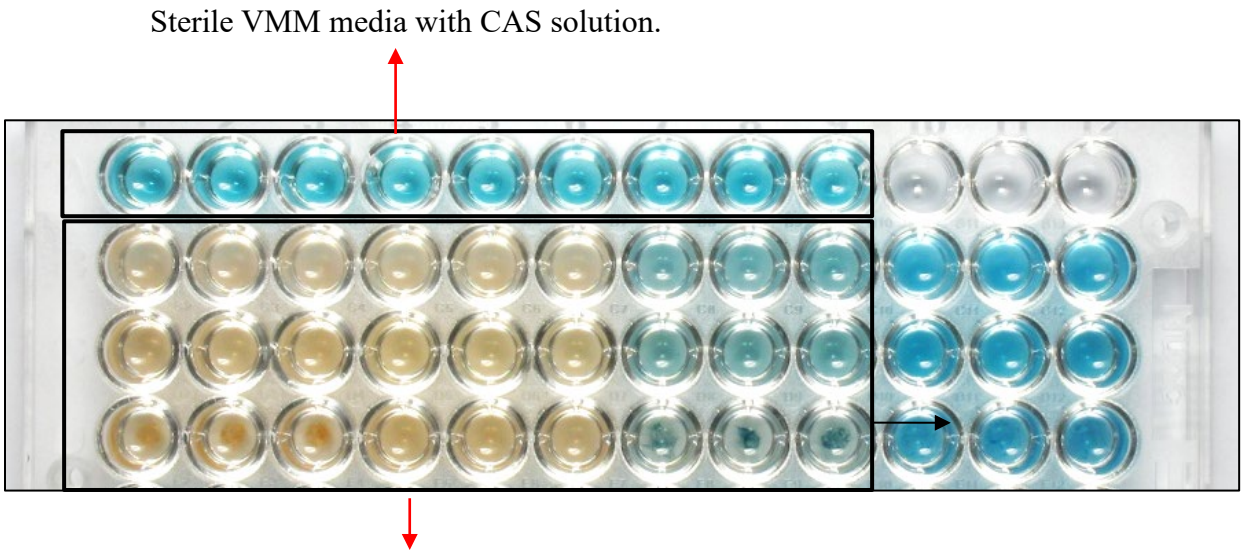

Figure 9. CAS liquid assay to detect siderophore production at 630 nm

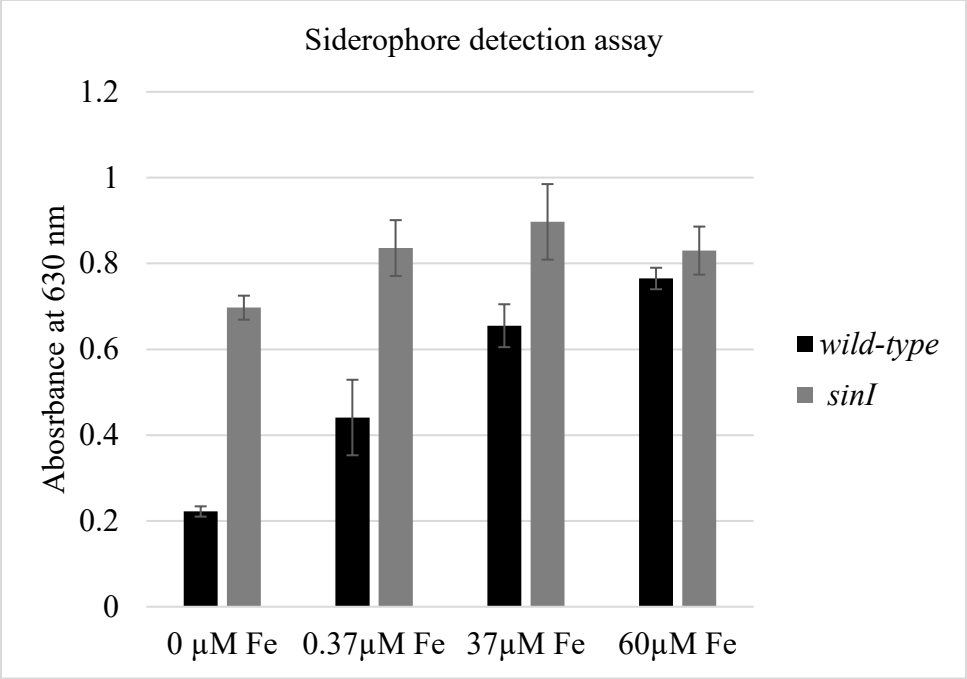

Figure 10. Siderophore activity measured by semi-quantitative liquid CAS assay where the Y-axis shows the absorbance at 630 nm. Black bars represent siderophore activity in a wild-type strains, gray bars represent in Rm8530*sinI*.

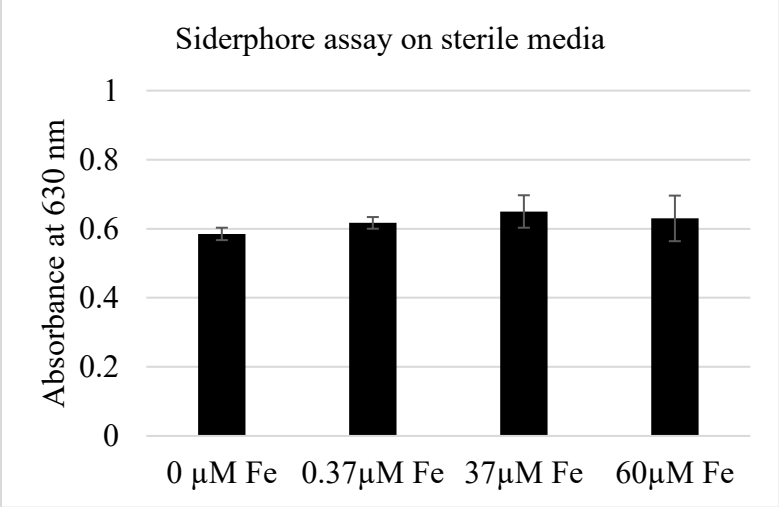

Figure 11. CAS assay on sterile VMM with varying concentrations of added iron chloride.

4.3 Differential regulation of rhizobactin 1021 biosynthesis genes in *S. meliloti*

Studies in the past have shown that the Sin locus and the ExpR regulator work in conjunction to control genes involved in the production of exopolysaccharides, motility, biofilm formation, symbiotic nitrogen fixation, transport of metals and small molecules (31). Therefore, to elucidate the role of the ExpR/Sin QS system on the rhizobactin regulon (Figure 5), we used qRT-PCR to measure the expression of the following genes: *rhbABCDEF*, *rhtX*, and *rhtA* in wild-type and QS mutant strains of *S. meliloti.* In all our gene expression studies, *Smc00128,* a housekeeping gene that is not regulated by QS, was used as our internal control (21). Results from the qRT-PCR analysis showed a dramatic decrease of up to ~40-fold in the expression of rhizobactin 1021 biosynthesis genes in the QS mutants when compared to the wild-type taken as the baseline (Figure 12). The QS mutants (Rm8530 *sinI* or Rm1021 *expR*) consistently displayed a lower expression of the rhizobactin 1021 genes, indicating that the presence of both *sinI* and *expR* component are required for full the expression of the rhizobactin 1021 biosynthesis and uptake genes.

To further investigate the activation properties of the ExpR/Sin QS system on the rhizobactin 1021 regulon, we measured the expression of *rhtA*, a gene that codes for the rhizobactin 1021 receptor. Data from the qRT-PCR analyses showed that the *rhtA* expression was ~50-fold higher in a wild-type strain when compared to the QS mutants (Figure 13). Similarly, we measured the expression of *rhrA*, a transcriptional activator of rhizobactin 1021 genes, and observed a significant difference of up to ~300-fold between a wild-type (8530) vs. a QS mutant (Figure 14). These results suggested that the ExpR/Sin system might regulate rhizobactin 1021 synthesis and

transport genes under iron-limiting conditions. Next, we wanted to further understand if the QS system acts upstream of the rhizobactin 1021 regulon to control the expression of these genes.

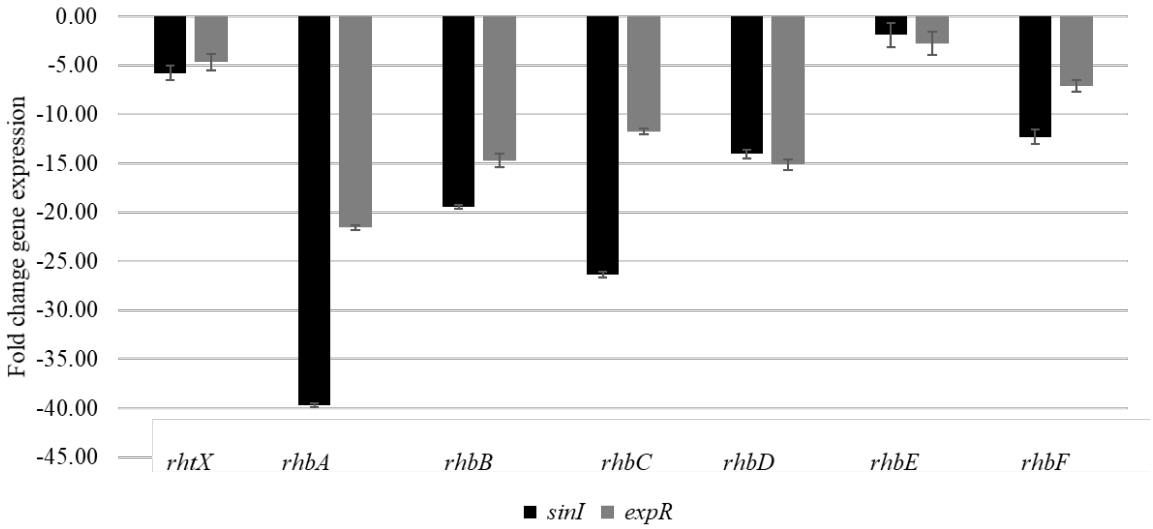

Figure 12. Relative gene expression of rhizobactin 1021. Negative values indicate the down-regulation of the denoted gene in the mutant strain compared to the wild-type taken as the baseline. The data are expressed as the mean of three independent biological replicates; error bars represent the standard deviation between three samples.

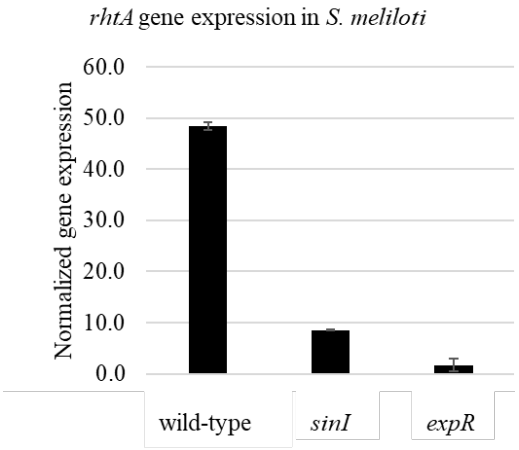

Figure 13. Normalized *rhtA* gene expression in the indicated strains. A student's *t*-test was conducted and a p-value<0.05 was obtained (p-value=0.0412). The data are expressed as the mean of three independent biological replicates; error bars represent the standard deviation between the samples.

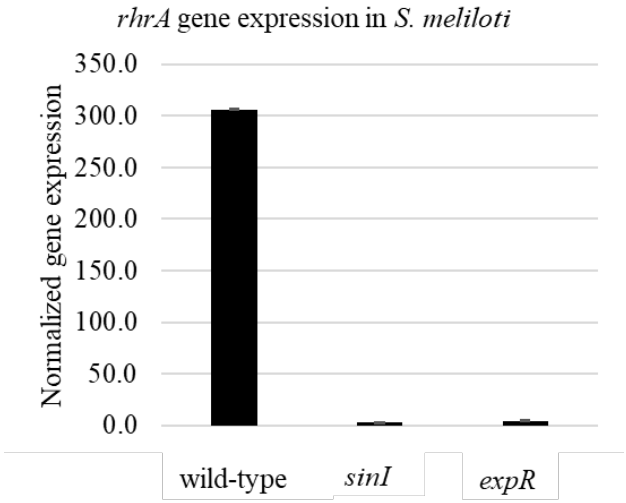

Figure 14. Normalized *rhrA* gene expression in the indicated strains. A student's *t*-test was and a **p*-value<0.05 was obtained. Results are the mean of three independent biological replicates; error bars represent the standard deviation between three samples.

4.4 Role of the ExpR/Sin QS system on *rhrA* in *S. meliloti*

To examine if the ExpR/Sin QS system acts directly or via *rhrA* to regulate rhizobactin 1021 genes, we next created mutants of *rhrA* in wild-type and QS mutant strain (Rm8530 *sinI*) of *S. meliloti* and quantified the expression of *rhbA* (first gene of the rhizobactin 1021 operon). The results from the qRT-PCR showed that the expression of *rhbA* in *rhrA* mutants was beyond the detectable limits (cut-off) of the assay (Figure 15). Similarly, liquid CAS assays performed on the *rhrA* mutants detected no siderophore activity suggesting that in the absence of *rhrA,* the synthesis and release of siderophores are abolished (Figure 16). Additionally, we also observed that a *sinI* mutant has the same effect as the *rhrA* mutant on the expression of *rhbA*. All these results taken together imply that the ExpR/Sin QS system plays a role in the regulation of siderophore synthesis via modulating the activity of *rhrA* under iron-limiting conditions. It is important to note that for our gene expression studies, cultures of *S. meliloti* were grown in minimal glutamate mannitol (MGM) low-

phosphate medium as described here (21). Despite the fact that this is minimal media, it is not completely iron free. Therefore, in all our future experiments that involved iron-restricted growth of *S. meliloti*, we used TY medium with added 2, 2'-bipyridyl to a final concentration of 200 µM (iron-limited complex medium) to maximize the conditions for siderophore biosynthesis (17). The increased restriction in iron provided by the TY media with added iron chelator resulted in a dramatic expression of *rhbA* gene as seen in a *sinI* mutant when compared to the data obtained in Figure 12.

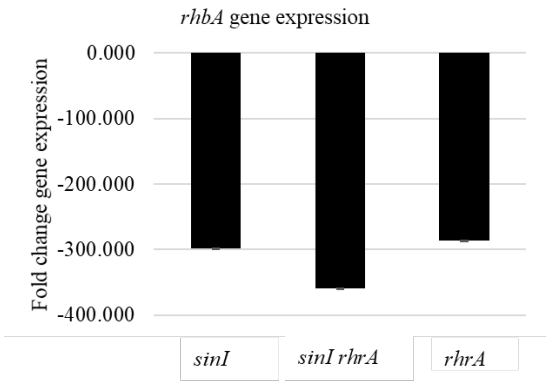

Figure 15. Fold change gene expression of *rhbA* in the indicated strains. Negative values indicate the down-regulation in the mutant strain compared to the wild-type taken as the baseline. Results are the mean of three independent biological replicates; error bars represent the standard deviation between three samples.

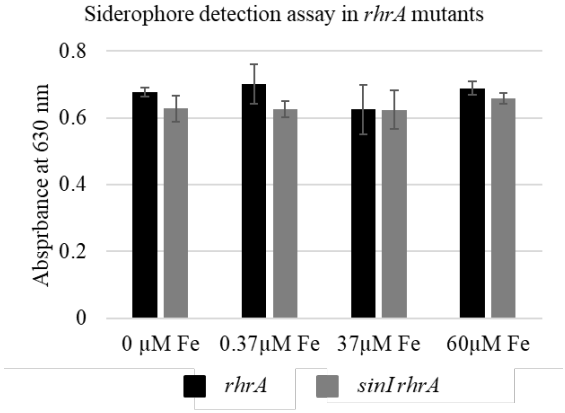

Figure 16. CAS assay in *rhrA* mutants grown in iron-replete and depleted conditions. 8530 *rhrA* is represented in black bars; *sinI rhrA* in gray bars.

4.5 A QS capable strain is more efficient in invading the root nodules compared to a mutant under iron-deficient conditions.

To establish a successful symbiosis and fix nitrogen for the plant, *S. meliloti* is not only required to perform the appropriate symbiotic functions at the right time, but it must also compete against other microbes in the rhizosphere for various resources (such as iron). Iron acquisition is essential for nitrogen fixation by the rhizobium-legume root nodule symbiosis (33). Therefore, to examine the effects of QS mediated siderophore production on nodulation efficiency, we inoculated germinated seedlings of *M. sativa* with wild-type and QS mutant strains (Rm8530 *sinI*) under iron-limiting conditions and measured the invasion efficiency.

Under low-iron conditions, plants inoculated with a QS wild-type strain mutant displayed a higher percentage of pink root nodules (80%) compared to the plants inoculated with a QS mutant strain Rm8530 *sinI* (40%) (Figure 17a). We also observed that plants inoculated with Rm8530 were, on average, approximately 4 cm taller and looked healthier than those inoculated with *sinI* mutant (Figure 17b). To understand if the addition of exogenous iron restores the inefficiency, we added 1% iron chloride to plant growth media and partially rescued the defect in nodulation efficiency in plants inoculated with a *sinI* mutant (Figure 17). These studies suggested that a wild-type strain is far more capable in invading the root nodules under iron-limiting conditions when compared to a QS mutant, implying that the presence of a QS system increases the ability of *S. meliloti* to cope with iron stress.

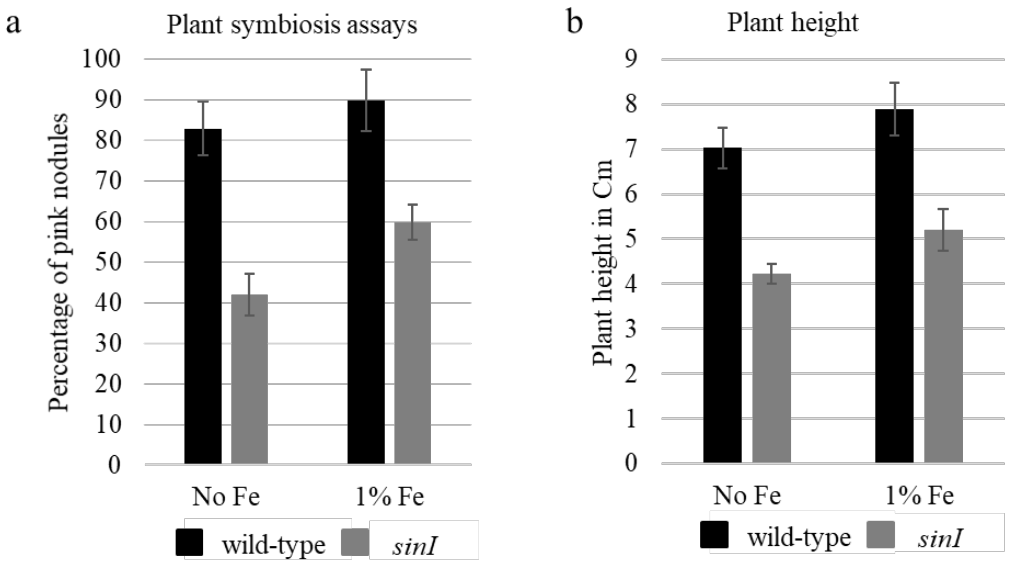

Figure 17. (a) *Medicago sativa* seedlings were inoculated with the indicated strains and grown on Jensen's agar plates with or without added iron. The results are the average of the total number of root nodules. (b) Plant height was measured in centimeters and recorded 4 weeks post-inoculation.

4.6 A QS mutant has the same effect as a *rhrA* mutant on the efficiency of root nodule invasion under iron-deficient conditions.

As we have discussed earlier, the demands for iron are very different between free-living rhizobia under natural conditions compared to rhizobia preparing to establish a symbiosis with the host plant (34). To determine if a QS mutation has the same effect as a *rhrA* mutation on the efficiency of root nodule invasion under iron-deficient conditions, seedlings of *Medicago sativa* were inoculated with *rhrA* mutants: Rm8530 *sinI rhrA*, Rm8530 *rhrA* and grown on Jensen's agar plates under iron-limiting conditions. Plants inoculated with *rhrA* mutants displayed a considerable defect in the efficiency of root nodule invasion when compared to the plants inoculated with wild-type (Figure 18a). In addition, we observed that a *sinI* mutant has the same effect as the *rhrA* mutant on plant root nodulation (Figure 18a). Similarly, plants inoculated with *rhrA* or *sinI* mutants

displayed poor growth and were approximately, 1-2 cm smaller in the average plant height when compared to the plants inoculated with the wild-type (Figure 18b). In summary, mutants of *rhrA* or *sinI* are inefficient in nodule invasion under iron-limiting conditions compared to wild-type.

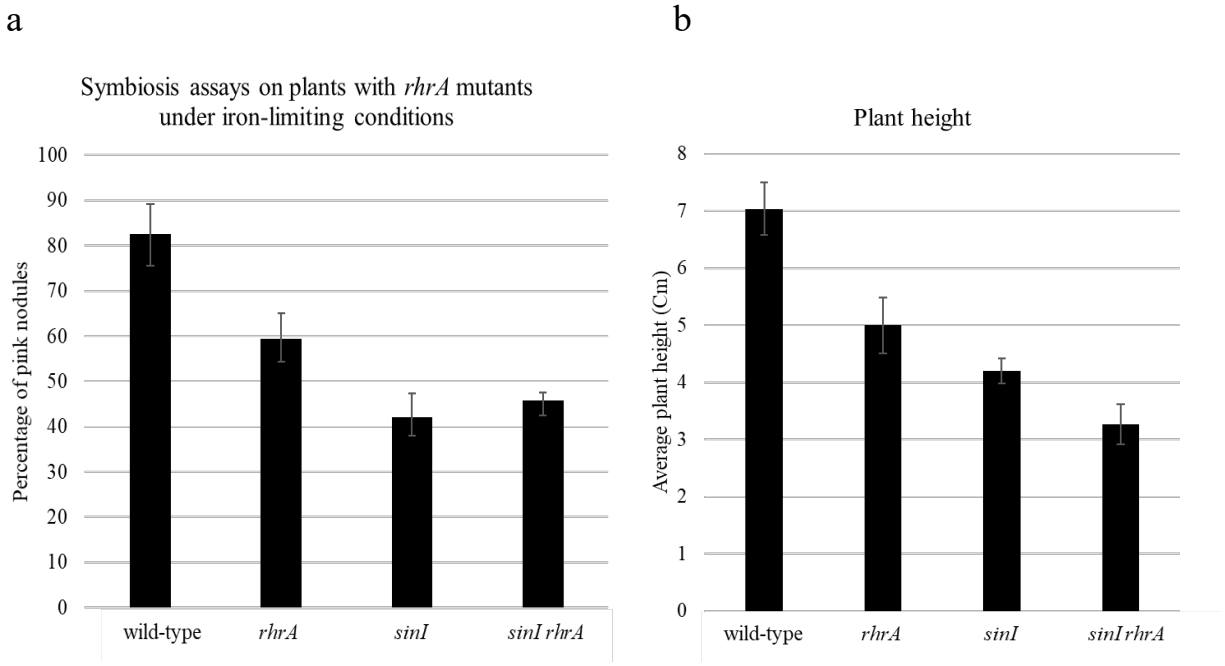

Figure 18. (a) Seedlings of *Medicago sativa* were inoculated with *rhrA* mutants and grown in Jensen's agar plates with no added iron. (b) Plant height was measured in centimeters and recorded 4 weeks post-inoculation. Data were obtained from an average of 75 plants in total per strain per condition.

4.7 Role of the ExpR/Sin QS system on RirA regulator

Previous reports have shown that RirA acts as a global regulator of iron-responsive genes in *R. leguminosarum* and *S. meliloti* (35). Biosynthesis and transport of rhizobactin 1021 were found to be negatively regulated in the presence of iron by RirA and positively regulated by transcriptional activator RhrA (35). To examine the role of the ExpR/Sin QS system on RirA repressor, we created mutants of *rirA* in *S. meliloti* and isolated RNA to quantify the expression of *rhbA* (first gene of

the rhizobactin 1021 operon). In the absence of *rirA*, the genes involved in rhizobactin 1021 synthesis were induced independently of the ExpR/Sin QS system (Figure 19). While the Sin system regulates rhizobactin 1021 biosynthesis via activating RhrA (as seen in our previous data), it does not seem to play a role in the repressor activity of RirA.

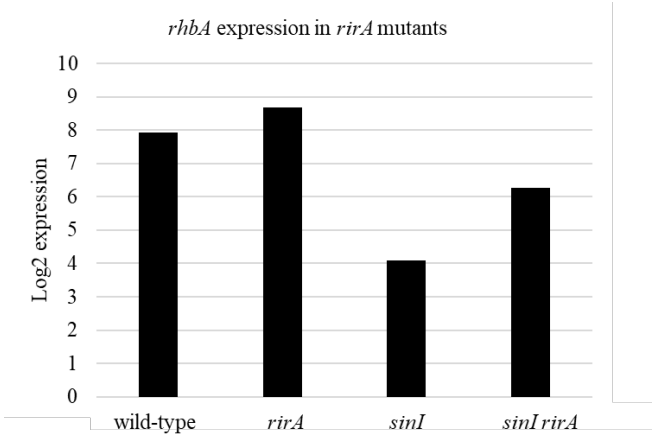

Figure 19. Log2 normalized gene expression values in *rirA* mutants in the indicated strains.

Similarly, plant symbiosis assays were conducted on seedlings of *M. sativa* inoculated with *rirA* mutants. Though we expected a *rirA* mutant to invade root nodules as efficiently as a wild-type, plants inoculated with *rirA* mutant displayed slight inefficiency in nodule invasion when compared to the plants inoculated with wild-type (Figure 20a). Among the plants inoculated with *sinI rirA*, the effect of SinI mutation was still prominent, as shown by the inefficiency in nodule invasion in addition to poor growth, when compared to the plants inoculated with *rirA* (Figure 20b). To summarize, plants inoculated with *rirA* were overall healthier and had a higher invasion efficiency under iron-limiting conditions, when compared to the plants inoculated with *sinI rirA*. These results suggest that RirA might have a wider role in global iron regulation in *S. meliloti* and

a mutation of *rirA* regulator may result in compounding effects on iron acquisition, which do not seem clear at this point of time.

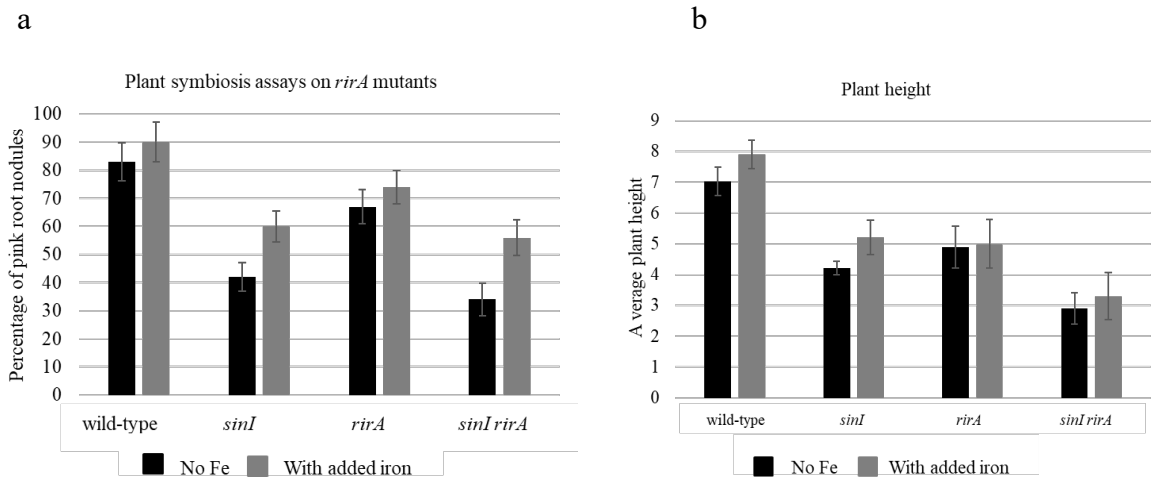

Figure 20. (a) **S**eedlings of *Medicago sativa* were inoculated with *rirA* mutants and grown in Jensen's agar plates with no added iron. (b) Plant height was measured in centimeters and recorded 4 weeks post-inoculation. Data were obtained from an average of 75 plants in total per strain.

CHAPTER 5

DISCUSSION

Sinorhizobium meliloti is a well-established model for understanding the role of QS in symbiotic nitrogen fixation. The ExpR/Sin quorum-sensing system in *S. meliloti* has been well studied for its role in root nodule invasion and other processes such as motility, chemotaxis, biofilm formation, and metal transport. Whole-genome-wide microarray analysis performed in our laboratory at different phases of the bacterial growth cycle identified over > 200 genes that were found to be controlled by the ExpR/Sin QS system. Some of them include the genes involved in siderophore synthesis and uptake, metal transport, heme transport, and Fe-S cluster synthesis (21). Additionally, the genes involved in the synthesis and transport of rhizobactin 1021 were found to be expressed in a wild-type strain at the mid-log phase of the growth cycle when the ExpR/Sin QS is active. However, this was not the case in mutants of *S. meliloti* missing either the *sinI* or the *expR* component of the QS system, suggesting a possible role of the ExpR/Sin QS system in siderophore synthesis. Therefore, in this study, the role of the ExpR/Sin QS system on siderophore-mediated iron uptake during symbiosis has been addressed.

qRT-PCR analyses of rhizobactin 1021 synthesis genes identified a significant reduction of up to ~40-fold in gene expression levels in strains lacking a functional QS system when compared to wild-type. The absence of either *sinI* or the *expR* component of the QS system was enough to affect the expression of rhizobactin 1021 genes (Figure 12). Moreover, liquid CAS assays to detect siderophore production revealed a 3-4-fold higher siderophore activity in a wild-type strain when compared to a QS mutant under iron-limiting conditions (Figure 10). When cells grown under iron-replete conditions were tested, no siderophore activity was detected (Figure 11).

As siderophore synthesis is a highly energy-driven process, bacteria do not synthesize siderophores when iron is abundant in the environment. Besides, under iron-rich conditions and in the presence of RirA, the genes involved in the synthesis and transport of rhizobactin 1021 are repressed (34). As an example of the effect of QS on rhizobactin 1021, we studied specifically the expression of *rhtA* and *rhrA*, the outer-membrane receptor of rhizobactin 1021, and a transcriptional activator of rhizobactin regulon respectively. Results from the qRT-PCR analyses indicated a dramatic difference ~50-fold in the expression of *rhtA* in a wild-type strain compared to the QS mutants (as seen in Rm8530 *sinI* and Rm1021 *expR*). These results indicated that the genes involved in siderophore synthesis and transport are not fully induced in the absence of an intact QS system (Figure 13). Similarly, qRT-PCR analyses identified a significant difference of up to ~300-fold in the expression of *rhrA* between a wild-type vs. a QS mutant under iron-limiting conditions (Figure 14).

To further understand if the ExpR/Sin system regulates siderophore biosynthesis genes by acting upstream of *rhrA*, we created mutants of *rhrA* and measured the expression of the genes involved in the synthesis and transport rhizobactin 1021. qRT-PCR analyses detected little to no expression of *rhbA* (first gene of the rhizobactin 1021 synthesis operon) in *rhrA* mutants (Figure 15). Likewise, liquid CAS assays detected no siderophore activity in *rhrA* mutants implying that under iron-limiting conditions, the biosynthesis of rhizobactin 1021 is dependent on the transcriptional activation by RhrA (Figure 16). Furthermore, a *sinI* mutant had the same effect as the *rhrA* mutant on the expression of rhizobactin 1021 genes. All these data taken together support our hypothesis that the ExpR/Sin QS system regulates siderophore synthesis and transport by modulating the activity of *rhrA*. Similar observations were reported by O'Connell *et al.*, where no

mRNA transcripts of *rhbA* and *rhtA* were detected in *rhrA* mutants under iron-liming conditions, suggesting that RhrA is an activator of both the siderophore synthesis and the transport genes [52].

As mentioned earlier, rhizobia preparing to establish a symbiosis with the host-plant have higher demands for iron vs. free-living rhizobia. Under iron-limiting conditions, free-living rhizobia uses several mechanisms such as the use of siderophores, ferric reductase, and heme to compete for iron scarcity (9). Contrastingly, bacteriods that have established a symbiotic association with host do not obtain iron via high-affinity iron uptake systems (11). Studies in the past have shown that mutations in ABC metal transporters, TonB receptors do not affect the established symbiosis (36). However, *S. meliloti* has increased nodule occupancy under iron-limiting conditions when compared to mutant strains with impaired siderophore uptake systems. Therefore, the ability to use siderophores for iron acquisition appears to provide a competitive advantage and impact the effectiveness of the legume-rhizobium symbiosis. We conducted studies on plants inoculated with a QS-capable strain vs. plants inoculated with a QS mutant grown under iron-limiting conditions and observed that a wild-type strain is far more efficient in invading the root nodules when compared to a QS mutant (Figure 17a). The inefficiency of the QS mutants compared to wild-type in nodule invasion can be partially restored when iron is added to the plant growth media, suggesting that the QS effect is due to low-iron conditions (Figure 17a & b).

To understand if the QS mutation has the same effect as *rhrA* mutation on the efficiency of root nodule invasion, seedlings of *M. sativa* were inoculated with *rhrA* mutants under iron-deficient conditions. Plants inoculated with *rhrA* mutants displayed a considerable defect in the efficiency of root nodule invasion when compared to the plants inoculated with wild-type (Figure 18a). In addition, we observed that a *sinI* mutant has a similar effect as the *rhrA* mutant on invasion

efficiency (Figure 18a). Moreover, plants inoculated with *rhrA* or *sinI* mutants displayed poor growth and were approximately, 2 cm shorter in the average plant height when compared to the plants inoculated with the wild-type (Figure 18b). To summarize, the mutants of *rhrA* or *sinI* are inefficient in nodule invasion when compared to wild-type under iron-limiting conditions. The addition of iron to the plant growth media partially rescued the defect in the phenotype.

The genes involved in the biosynthesis and transport of rhizobactin 1021 are positively regulated by RhrA and negatively in the presence of iron (35). RirA acts as a repressor of the rhizobactin 1021 regulon in addition to other iron response genes in the presence of iron. Therefore, RhrA is not only induced by iron-limitation but also in the absence of RirA. To understand the role of the ExpR/Sin system on the repressor activity of RirA, we created mutants of *rirA* in *S. meliloti* and measured the expression of rhizobactin 1021 synthesis genes. Our qRT-PCR data showed that in the absence of *rirA*, the genes involved in the synthesis of rhizobactin 1021 are derepressed independently of the ExpR/Sin QS system (Figure 19). While the Sin system regulates rhizobactin 1021 biosynthesis via RhrA, it does not seem to play a role in the repressor activity of RirA (figure 7).

Symbiosis assays conducted on plants inoculated with *sinI rirA* mutants displayed poor growth and formed fewer pink nitrogen-fixing root nodules when compared to the plants inoculated with *rirA* (Figure 20a). We expected a *rirA* mutant to be as efficient or better than wild-type in invading the root nodules. However, plants inoculated with *rirA* were slightly inefficient in nodule invasion when compared to the plants inoculated with wild-type (Figure 20a). Nevertheless, plants inoculated with *rirA* were much healthier and displayed an overall higher nodulation efficiency when compared to the plants inoculated with *sinI rirA* (Figure 20b). The

addition of iron chloride to the plant growth media partially rescued the defective phenotype. It is likely that RirA has a wider role in global iron regulation, and mutating *rirA* seems to have other effects that remain unclear at this stage.

All the results from plant symbiosis studies taken together suggested that *S. meliloti* with a functional ExpR/Sin QS system has higher nodulation efficiency under iron-limiting conditions when compared to strains defective in a high-affinity iron uptake system. Siderophore mediated iron acquisition regulated by the ExpR/Sin quorum-sensing system appears to provide a competitive advantage to bacteria in the rhizosphere to obtain the necessary iron resources for nodulation. Moreover, the analyses of these strains in iron-limiting conditions provided us with a deeper understanding of the regulation of siderophore biosynthesis and transport genes by the ExpR/Sin quorum-sensing system. Previous studies have shown that the genes involved in the biosynthesis or transport of rhizobactin 1021 are not expressed in the root nodules 5-weeks post-inoculation (11). Therefore, the process of siderophore-mediated iron acquisition appears to be a crucial step for the bacteria to reserve enough iron resources in the initial steps of nodulation. The ExpR/Sin QS mediated rhizobactin 1021 regulation is a likely factor that contributes to the effective competitiveness of *S. meliloti* in iron-limiting conditions.

In this current study, we propose a model for ExpR/Sin mediated siderophore uptake in *S. meliloti* (shown in Figure 21). Under iron-limiting conditions, the ExpR/Sin QS system upregulates the genes involved in the biosynthesis and transport of rhizobactin 1021 via *rhrA*, resulting in the synthesis and release of rhizobactin 1021 outside of the cell. The iron-bound siderophore is then transported into the cell cytoplasm via a complex network of cognate outer-membrane receptors, transmembrane proteins, permeases, periplasmic binding proteins, and ABC

metal transporters. Once inside the cell, the ferric form of iron is reduced to the ferrous form, and the iron is incorporated into various proteins.

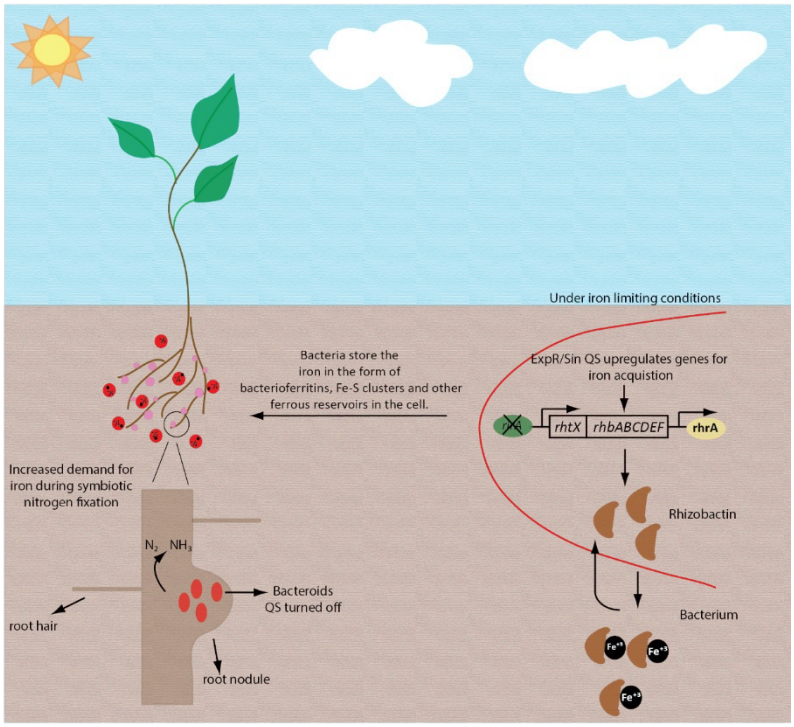

Figure 21. Putative model for the role of QS in siderophore mediated iron uptake during symbiosis. Under conditions of strict iron limitation, the ExpR/Sin QS up-regulates the genes involved in iron acquisition via *rhrA* resulting in the synthesis and release of rhizobactin 1021, a predominant siderophore produced by *S. meliloti*. Rhizobactin 1021 binds to the ferric form of iron in the environment, and via cognate receptors, the iron-bound rhizobactin 1021 is transported into the cell.

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BIOGRAPHICAL SKETCH

Nymisha Avadhanam was born in India, on December 15th, 1986, to A. Raghu Kumar and A. Nagamani. After completing her schooling in 2004, she joined the Department of Biotechnology, JNTU for her undergraduate studies. In 2009, she moved to the USA to pursue her master's in biological sciences from UT Arlington. After receiving her master's degree in 2011, she joined as a Clinical Research Fellow at UT Health Science Center/Veterans Health Administration. In 2013, she was accepted into the molecular and cell biology program at UT Dallas, Richardson, and began attending classes in August 2013. She received her Master of Science in Molecular and Cell Biology and qualified as a doctoral candidate in 2015.

CURRICULUM VITAE

Nymisha Avadhanam

EDUCATION

Doctor of Philosophy in Cell and Molecular Biology, University of Texas at Dallas, 2019

Master of Science in Cell and Molecular Biology, University of Texas at Dallas, 2015

Master of Science in Biology, University of Texas at Arlington, 2011

Bachelor of Technology in Biotechnology, JNTU, 2012

RESEARCH EXPERIENCE

Doctoral Researcher, Department of Biological Sciences, UT Dallas 2013-2019

- Microbiology skills: media preparation, bacterial cell culture in different media, phage-mediated transductions, and aseptic techniques.
- Molecular Biology skills: DNA/RNA/Protein isolation and quantification, vector-based cloning, PCR, gel agarose electrophoresis, qRT-PCR.
- Responsible for general safety of the lab. Duties included waste disposal, knowledge of laboratory stocks, strains, and maintaining laboratory supplies.
- Experienced in the areas of microbiology, microbial genetics, bacterial cell-cell regulation, and biochemistry.

Graduate Teaching Assistant

- Organizing Biochemistry, and General Microbiology laboratory courses.
- Duties included demonstrating lab techniques, monitor student participation, supervise undergraduate and fellow graduate teaching assistants.
- Grade exams, quizzes, home-works, and weekly lab reports.

Clinical Research Fellow, UT Health Science Center, San Antonio 2011-2013

Project I: Host genetic determinants of HIV-AIDS pathogenesis and gene regulation of CCR5

- To understand the role of genetic and epigenetic modifications in the susceptibility of HIV/AIDS pathogenesis.
- To analyze DNA methylation changes in various host determinants of HIV/AIDS.

Project II: Allergen Challenge Chamber Clinical Trial

- To discover biomarkers for allergy associated immune responses in patients using blood and nasal swab samples pre and post-exposure to allergens.
- Expertise in the following techniques: Next Generation Sequencing library preparation, mammalian cell culture, Bisulfite Conversion, DNA methylation Sequencing, Chromatin Immunoprecipitation, and microRNA isolation.
- Data Analysis and Bioinformatics tools: SDS, UCSC, and CLC.
- Instrumentation: Illumina HiSeq2000, CBot Cluster generation, Agilent Bioanalyzer, Quantitative/Real-Time PCR, TECAN, Illumina Iscan for Microarrays, and GWAS, Qubit, AutoMACS Pro Cell Sorting Technology, Bioruptor, automated IP Diagenode.

Junior Research Fellow (JRF), IICT, Government of India 2007-2008

Worked on "Finding Novel drug targets for *Trypanosoma cruzi* using a Computational Approach", IICT (Indian Institute of Chemical Technology) under the supervision of Dr. U. S. N. Murty, Scientist-F/Deputy Director, Head–Biology Division, Coordinator, Indian Institute of Chemical Technology (IICT), CSIR.

- Experience on various bioinformatics tools like BLAST, FASTA, PAM, BLOSSOM, CastP Server, ATLAS, alignment tools, homology modeling, RASMOL.
- Determination of protein structure validation and testing stereochemical quality of proteins.
- Directed the project to the discovery of potential drug molecules, clinical testing, and targeted drug delivery using the novel sites.

ACCOMPLISHMENTS

Conferences

- Presented at American Society for Microbiology (ASM) Annual Texas Fall Chapter, 2015 and 2016 on "Control of iron regulation and uptake by quorum sensing in *S. meliloti*".
- Organized the 2019 Annual Royston C. Clowes Lecture Series to honor the late Dr. Royston C. Clowes, former Head of the Molecular and Cell Biology program at UT Dallas.
- Presented at World Allergy and Asthma Congress, 2013, Milan, Italy, on "Evaluation of ultra-rush immunotherapy for Virginia live oak in a pollen chamber identifies cytokine and transcriptional correlates of protection", June 2013.
- Poster presentation at 15th Annual Medicine Research Day, 2012, organized by University of Texas Health Science Center at San Antonio on "Whole Genome Transcriptional Profiling of RNAseq Data on Differential T-Cell Activation in Humans and Chimps".
- Presented at Third Annual Frontiers of Translational Science Research Day, 2012 at University of Texas Health Science Center at San Antonio on "Whole Genome

Transcriptional Profiling of RNAseq Data on Differential T- Cell Activation in Humans and Chimps".

- Attended a 3-day international conference on "Agri-Biotechnology for Sustainable Agriculture" held during 9th-11th March 2007 at Pragathi Resorts, Hyderabad.
- Attended a 3-day workshop on "Design of Experiments for Scientists and Engineers" at Indian Institute of Chemical Sciences (IICT, CSIR).

Publications

1) Iron Uptake and Regulation in *Sinorhizobium meliloti*, Ph.D. Thesis, University of Texas at Dallas, Richardson, TX, 2019.
2) Control of iron regulation and uptake by the ExpR/Sin QS system in *Sinorhizobium meliloti*, N Avadhanam, JE González (to be submitted to The Journal of Bacteriology).
3) Epigenetic mechanisms, T-cell activation, and CCR5 genetics interact to regulate T-cell expression of CCR5, the major HIV-1 coreceptor, German G. Gornalusse, Srinivas Mummidi, Nymisha Avadhanam, Robert A. Clark, Weijing He, and Sunil K. Ahuja., Proceedings of the National Academy of Sciences (PNAS), August 2015 (selected for the 'In This Issue' highlights (PNAS 112(34):10563-64) by PNAS Editors.
4) Evaluation of ultra-rush immunotherapy for Virginia live oak in a pollen challenge chamber identifies cytokine and transcriptional correlates of protection, Jacobs, RL; Harper, N; He, W; Rather CG; Castiblanco, J; Carrillo, A; Manoharan, M; Avadhanam, N; Liu, Y; Ingale, P; Andrews, CP; Ramirez, DA; Ahuja, SK, Allergy, 2013.

Certifications

- Writing in Sciences, Stanford University, 2018.
- Initiating and Planning Projects (Project Management), University of California, Irvine, 2017.
- Introduction to Genomic Technologies, Johns Hopkins Bloomberg School of Public Health, 2017.

Honors and Awards

- Samuel Kaplan Award, Certificate of Outstanding Scientific Achievement in Medical/Environmental Microbiology, by American Society for Microbiology (ASM) Texas Fall Chapter, 2015.
- Listed on the Dean's booklet for the Academic Excellence Week 2010, UTA on SOAR Tutor Level I & II Certification Program, which aims in tutoring and mentoring undergraduate students in the field of Science and Engineering.
- Best Outgoing Student Award 2006-2007, Department of Biotechnology, JNTU.

- State 5th rank and All India 106th rank in National Science Olympiad, 2003.

Organizations I am a member of:

- American Association for the Advancement of Science (AAAS)/ Science Program for Excellence in Science.
- Biological Sciences Graduate Student Association (BSGSA), UT Dallas.
- Phi Sigma Graduate Honor Society, Beta Phi Chapter, Department of Biological Sciences, UT Arlington.