Chapter 3
Mechanisms and Regulation of Iron Homeostasis in the Rhizobia

Elena Fabiano and Mark R. O'Brian

Abstract Rhizobia are soil bacteria belonging to different genera whose most conspicuous characteristic is the ability to establish a symbiotic association with legumes and carry out nitrogen fixation. The success of these organisms in the rhizosphere or within the host plant involves the ability to sense the environment to assess the availability of nutrients, and to optimize cellular systems for their acquisition. Iron in natural habitats is mostly inaccessible due to low solubility, and microorganisms must compete for this limited nutrient. In addition to their agricultural and economic importance, rhizobia are model organisms that have given new insights into related, but less tractable animal pathogens. In particular, genetic control of iron homeostasis in the rhizobia and other α-Proteobacteria has moved away from the Fur paradigm to an iron sensing mechanism responding to the metal indirectly. Moreover, utilization of heme as an iron source is not unique to animal pathogens, and the rhizobial strategy reveals some interesting novel features. This chapter reviews advances in our understanding of iron metabolism in rhizobia.

Keywords Iron acquisition · Heme · Rhizobial–legume interactions · Metalloregulation

Rhizobia are a diverse group of Gram-negative soil bacteria that can form a symbiosis with leguminous plants. In general, a rhizobial species recognizes one or

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a few legume hosts; the molecular basis for the specificity between the bacterium and host, and the events leading to the symbiotic state are understood in considerable detail (Downie and Walker 1999; Oke and Long 1999; Spaink 2000; Gibson et al. 2008; Oldroyd and Downie 2008; Masson-Boivin et al. 2009). Rhizobia elicit the formation of a symbiotic organ called a root nodule comprising differentiated plant and bacterial cells. In this context, rhizobia are endosymbionts within the plant cells of the nodule.

Differentiated rhizobia within nodules are termed bacteroids, and acquire the ability to convert atmospheric nitrogen into ammonia by a process called nitrogen fixation. Nitrogen fixed by the endosymbiont is exported out of the cell in the form of ammonium or amino acids, and subsequently assimilated by the plant host to fulfill its nutritional nitrogen requirement. In return, the plant provides the bacteroids with a carbon source ultimately derived from photosynthesis. The rhizobia-legume symbioses account for nearly half of the global biological nitrogen fixation (Gruber and Galloway 2008), and is therefore an important agricultural and environmental process. Moreover, the rhizobia are phylogenetically related to several plant and animal pathogens, and serve as a model to understand the molecular basis of bacterial–eukaryote interactions.

Rhizobia include the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium,* and *Allorhizobium*. These genera belong to the α-Proteobacterial subdivision of the purple bacteria, an extremely diverse group that includes pathogens, symbionts, photosynthetic organisms, bacteria that degrade environmental pollutants, and the abundant marine organism *Pelagibacter ubique* (Ettema and Andersson 2009). The bacterial ancestor of mitochondria belongs to this group as well. More recently, numerous α-Proteobacterial species have also been identified that form symbiosis with legumes (Moulin et al. 2001; Chen et al. 2003; Taulé et al. 2012). Although these bacteria are also referred to as rhizobia, they are phylogenetically distinct from the α-Proteobacterial species, and are not considered further here.

In recent years, studies on rhizobial iron metabolism have focused to a large extent on three species, *Rhizobium leguminosarum*, *Sinorhizobium meliloti* and *Bradyrhizobium japonicum*. *R. leguminosarum* and *S. meliloti* belong to the family Rhizobiaceae, whereas *Bradyrhizobium* belongs to the *Bradyrhizobiaceae*. These two families diverged approximately 500 million years ago (Turner and Young 2000), and thus it is not unexpected that important differences between them have been observed with respect to iron metabolism.

3.1 The Problem of Iron Acquisition and Roles of Iron in Symbiosis

As a free-living organism, rhizobia must compete with other soil microbiota for nutrients and other resources, including iron. Iron is an abundant element, but it is

primarily in the oxidized form, which has an extremely low solubility in aqueous environments at neutral pH. As described below, bacteria have developed numerous strategies to acquire iron from the environment, including highjacking other microbial iron acquisition systems. At present, much more is known about iron acquisition and regulation in free-living rhizobia than in symbiosis.

Bacteroids within a functional nodule are surrounded by the peribacteroid membrane, a plant structure which separates the bacteria from the host within the plant cell. The peribacteroid membrane encompasses one or many bacteroids, depending on the type of nodule formed by the plant, and this structure is called a symbiosome. This means that the supply of iron and other nutrients to the bacteria is ultimately controlled by the plant. Whereas bacteria in the rhizosphere likely experience iron stress due to the low solubility of ferric iron, it cannot be assumed that the symbiotic prokaryote is iron-limited despite the large demand. At present, the mode of transport of iron into bacteroids is not known, nor is it known whether a high affinity transporter is necessary. Evidence for an iron pool in the peribacteroid space of soybean nodules could suggest that the bacteroids perceive a high iron environment (LeVier et al. 1996), but none of these issues have been resolved.

Iron is a major constituent of two crucial proteins for the N_2-fixing symbiosis process, bacterial nitrogenase and plant leghemoglobin. Moreover, other iron containing proteins which contribute to the N_2 fixation process are induced symbiotically, such as rhizobial hydrogenase and cytochromes that allow respiration in the low oxygen milieu of the nodule (Hennecke 1992; Sangwan and O'Brian 1992; Baginsky et al. 2002).

The nitrogenase complex contains about 34 atoms of iron. It comprises the iron protein (the nucleotide binding and electron-donating element) and the iron–molybdenum protein which contains the N_2-reducing site. The iron protein is a homodimer of two subunits that coordinate one [4Fe-4S] cluster. The iron–molybdenum protein is a tetramer of two polypeptides ($\alpha_2\beta_2$), where the α subunit contains a [Mo-7Fe-9S-homocitrate] cluster and the β subunit a [8Fe-7S] cluster (Georgiadis et al. 1992; Rees and Howard 2000). Nitrogenase constitutes about 30 % of the soluble proteins (11 % of total proteins) in the bacteroid (Verma and Nadler 1984).

In N_2-fixing nodules, leghemoglobin can represent about 20 % of the total nodule iron (1–3 mg of leghemoglobin per gram of fresh weight depending on plant species) (Dilworth 1980). As previously mentioned, differentiated bacteroids are enveloped in a peribacteroid membrane and immersed in a leghemoglobin-rich environment. It is interesting to note that peribacteroid membrane is one of the first structures to be degraded during the nodule senescence (Herrada et al. 1993). In this scenario, it is possible that iron requirement is greater for nodulated plants than for host plants alone. In fact, it has been reported that iron deficiency negatively affects the rhizobia–legume symbiosis. Application of the metal to iron-stressed plants increases nodule number and plant mass. Furthermore, *S. meliloti* iron-starved cells are less competitive for nodulation, and iron deficiency prevents nodule initiation and limits nodule development (O'Hara et al. 1988; Tang et al. 1990; Expert and Gill 1992; Battistoni et al. 2002b).

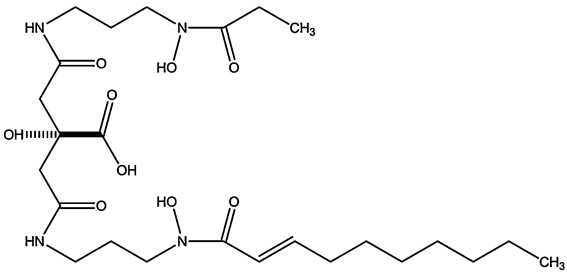
3.2 Iron Transport Systems

3.2.1 Siderophore-Mediated Iron Transport

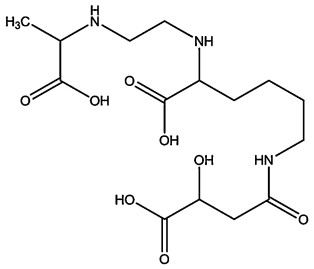
A common strategy used by many bacteria to obtain iron from their environment when it is scarce involves the production and secretion of ferric chelating compounds termed siderophores (Greek for iron carriers) (Lankford 1973; Neilands 1973, 1981; Ratledge and Dover 2000). Siderophores are low molecular weight compounds (around 300–1,500 Da) that bind iron specifically and with high affinity. The secreted siderophore, bound to iron, is then taken up by the cell through a ferric siderophore transport system. In Gram-negative bacteria, translocation of ferric siderophore complexes across the outer membrane requires energized transport via outer membrane receptors. The energy is provided by the proton motive force, and it is transduced to outer membrane receptors by the TonB-ExbB-ExbD complex (Postle and Kadner 2003).

Although siderophores differ widely in their overall structure, variation in metal-binding groups is more limited. According to the iron-chelating group, siderophores are classified as catecholates, hydroxamates, α-hydroxycarboxylates, or mixed siderophores. Although these are the most common groups, other metal-binding groups have been identified, for example oxazoline, thiazoline, hydroxypyridine, and *β*-hydroxyacids. Siderophores can also be characterized according to their biosynthetic mechanism as nonribosomal peptide synthetase (NRPS)-dependent or NRPS-independent (Raymond and Dertz 2004; Challis 2005; Donadio et al. 2007).

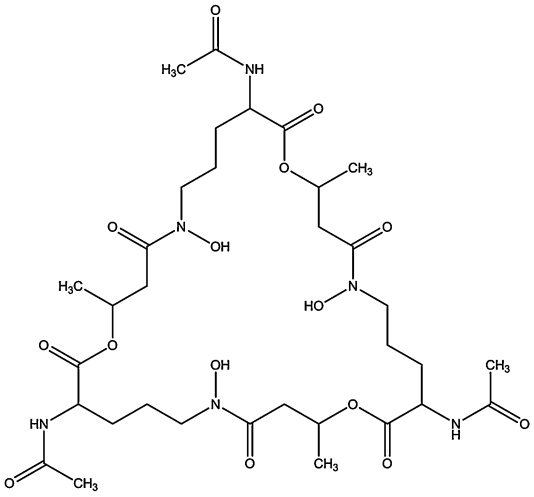
Three rhizobial endogenous siderophores have been chemically characterized to date (Fig. 3.1): (i) Vicibactins, trihydroxamate NRPS-dependent siderophores synthetized by *R. leguminosarum.* (ii) Rhizobactin, and α-hydroxycarboxylate siderophore produced by *S. meliloti* strain DM1 (iii) Rhizobactin 1021, a citrate-derivative di-hydoxamate and NRPS-independent siderophore syntethized by *S. meliloti* strain 1021 (Persmark et al. 1993; Dilworth et al. 1998; Carter et al. 2002; Smith et al. 1985). Siderophore biosynthesis has been studied in *R. leguminosarum* 8401(pRL1JI) and *S. meliloti* 1021. In both cases, biosynthetic genes are located on plasmids and are clustered close to their respective outer membrane receptors (Fig. 3.2). In addition, some *R. leguminosarum* species produce trihydroxymates, while some sinorhizobia produce dihydroxymates or α-hydroxycarboxylates although chemical structures for them are unavailable (Carson et al. 2000). Catechol-like siderophore production has been reported in *R. leguminosarum* IARI 102 and *Rhizobium sp* (cowpea) RA-1 (Modi et al. 1985; Patel et al. 1988). Unfortunately, none of these structures have been resolved. Anthranilate, a precursor of tryptophan, has been reported to promote the uptake of ferric iron into iron-starved cells in *R. leguminosarum* (Rioux et al. 1986). Anthranilate was synthesized and transported in cultures grown with iron, and therefore is not a specific response to iron deficiency. Anthranilate synthesis mutants of *S. meliloti* form defective nodules that cannot be accounted for by a tryptophan auxotrophy



Rhizobactin 1021. The siderophore produced by *S. meliloti* 1021.



Rhizobactin. The siderophore produced by *S. meliloti* DM4



Vicibactin. The siderophore produced by *R. leguminosarum* WSM710

since downstream mutants in the tryptophan pathway form effective nodules (Barsomian et al. 1992). Interestingly, the *B. japonicum* 110 genome is a single chromosome with no plasmids, and no endogenous siderophore has been identified in that species. Nevertheless, *B. japonicum* does have genes encoding siderophore receptors, as discussed below.

Fig. 3.1 Chemical structures of three siderophores characterized in rhizobia

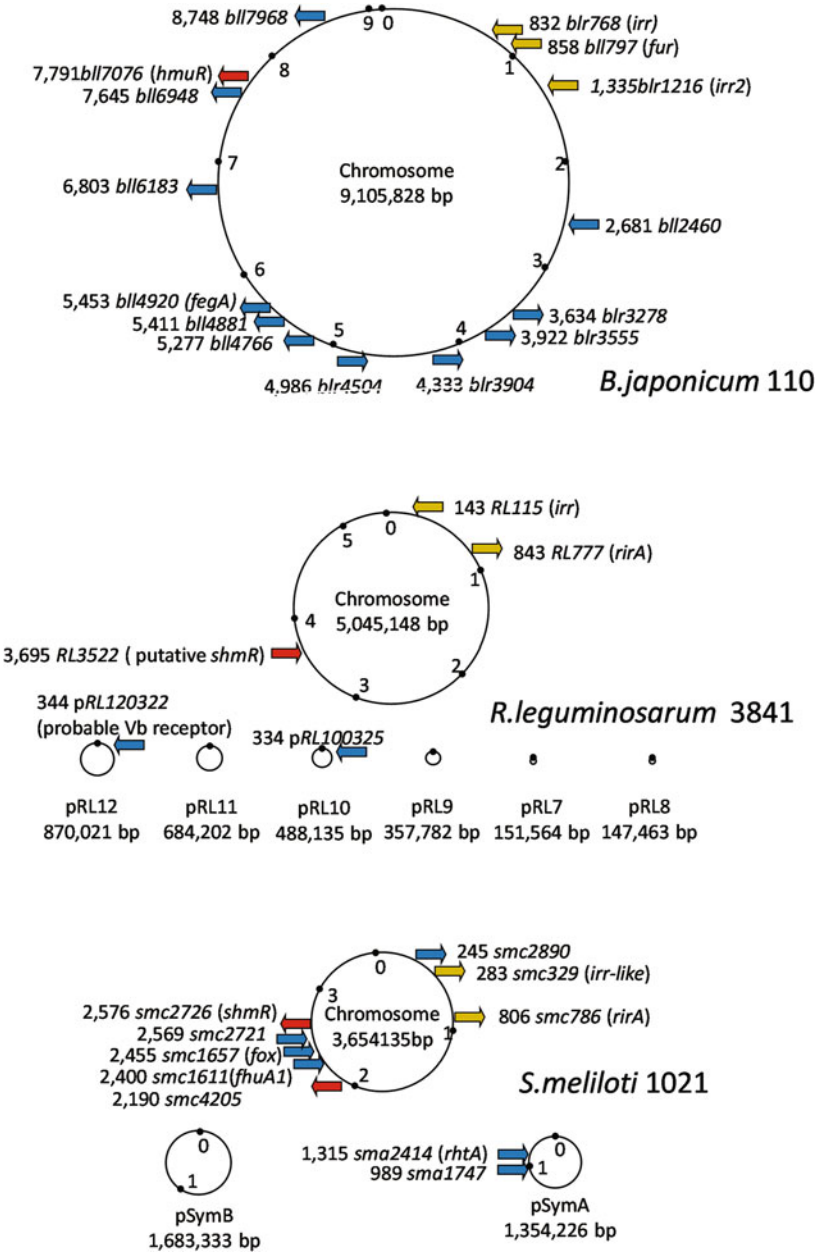


Fig. 3.2 Genetic maps of genes coding putative TonB-dependent receptors and iron regulators in *B. japonicum* 110, *R. leguminosarum* 3841, and *S. meliloti* 1021. Genes coding heme receptors are shown as *gray arrows*, other putative TonB-dependent receptor genes as *black arrows*, and iron regulator genes as empty *arrows*. The directions of transcription are indicated by the *arrows*. The distances in Mbp from the origins of replication are indicated as *dots*. The approximate locations of the genes are shown in kbp before the name of the genes. Sizes of chromosomes are indicated into the *circles*, and sizes and names of plasmids at the *bottom* of each plasmid

3.2.1.1 Vicibactin

Vicibactin, the siderophore made by *R. leguminosarum*, is a cyclic trihydroxamate containing three residues each of D-3-hydroxybutanoic acid and *N2*-acetyl-*N5*-hydroxy-D-ornithine arranged in alternate ester and peptide bonds (Dilworth et al. 1998) (Fig. 3.1). Vicibactin was identified from three different *R. leguminosarum* strains, WSM709, WSM710, and WU235. The sequenced *R. leguminosarum* 3841 strain also contains genes presumably involved in vicibactin biosynthesis located on plasmid pRL12 and close to the putative vicibactin receptor (Fig. 3.2).

Vicibactin biosynthesis involves an NRPS gene homolog, *vbsS* (Carter et al. 2002). NRPSs are assembly lines of specialized domains that form peptide bonds independently of ribosomes (Finking and Marahiel 2004; Donadio et al. 2007). NRPSs use amino or hydroxy-acids as building blocks, catalyzing the formation of amide or ester bonds, respectively. Each NRPS module consists of three basic domains: an adenylation domain (A) that activates the selected amino acid as an acyl adenylate, a peptide carrier (PCP) or T domain responsible for the formation of the thioester bond, and a condensation domain (C) which catalyzes peptide bond formation between two activated amino acids elongating the peptide and translocating it to the PCP domain. These three domains form a module, and NRPSs are consecutive tandems of these modules with each module being responsible for the addition of a new amino acid. Modules are colinear with the amino acids they link. A final module containing a thioesterase (TE) domain, instead of the C domain, completes the peptide formation. Additional activities may be catalyzed by specialized domains such as cyclization (Cy domain), epimerization (E domain), methylation (M domain), or oxidation or reduction (Finking and Marahiel 2004; Donadio et al. 2007).

Biosynthesis of vicibactin involves a cluster of eight genes arranged in four operons, *vbsGSO*, *vbsADL*, *vbsC,* and *vbsP* (Dilworth et al. 1998; Carter et al. 2002). A biosynthetic pathway has been proposed based on some mutant phenotypes and in silico studies. According to the model, the first step in biosynthesis is the hydroxylation of L-ornithine to give LGENES ARE MAXIMALLY EXPRESSED-N^5-hydroxyornithine probably catalyzed by VbsO. L-N^5-hydroxyornithine is then the substrate for VbsS, an enzyme similar to NRPS. VbsS activates the substrate by adenylation and transfers it to the PCP domain. The location and sequence of *vbsP* suggests that VbsP is responsible for the addition of phosphopantotheinate to the PCP domain, although this gene is not essential probably due to the presence of another phosphopantetheinyl transferase. The addition of butyryl coenzyme A to hydroxyornithine attached to VbsS, may be then catalyzed by VbsA. The resulting N^5-hydroxy-N^5-(D-3-hydroxybutyryl)-ornithine could be subsequently epimerized by VbsL. After the acetylation catalyzed by VbsC, the N^2-acetyl-N^5-hydroxy-N^5-(D-3- hydroxybutyryl)-ornithine bound as a thioester to the PCP domain of VbsS may be trimerized and cyclized by the TE domain of VbsS. The *vbsG* gene is essential for vicibactin biosynthesis although its function is still unknown. Based on sequence similarity, it has been suggested that the *vbsD* gene could be involved in siderophore export, although it is not essential in *R. leguminosarum*as *vbsD* mutants that accumulate normal amounts of siderophores (Yeoman et al. 2000). Vicibactin secretion is a topic that requires further investigation.

In *R. leguminosarum* 8401(pRL1JI) a gene, *fhuA1*, similar to the ferrichrome receptor gene from *E.coli,* is found adjacent to the *vbs* genes (Yeoman et al. 2000). In *E. coli*, the ferrichrome uptake system is encoded by the *fhuACDB* operon. FhuA is the outer membrane receptor, while FhuBCD is the ABC transport system involved in ferrichrome uptake: FhuB is a permease, FhuC an ATP-binding cassette protein, and FhuD the periplasmic protein (Burkhardt and Braun 1987; Coulton et al. 1987; Koster and Braun 1990). Upstream and transcribed in the same orientation as *fhuA1*, is located a gene, *fhuF,* whose predicted protein is similar to the *E. coli* putative ferric reductase. It has been suggested that it may be involved in iron release from the ferric-vicibactin complex. The *fhuCDB* operon is predicted to encode a periplasmic vicibactin-binding protein (FhuD), an inner membrane transporter (FhuB), and an ATPase (FhuC) involved in vicibactin transport through the inner membrane (Stevens et al. 1999). Vicibactin internalization requires a functional TonB protein (Wexler et al. 2001).

3.2.1.2 Rhizobactin 1021

The siderophore produced by *S. meliloti* 1021 was named rhizobactin 1021 to differentiate it from rhizobactin, an amino polycarboxylic acid siderophore produced by *S. meliloti* DM4 (Persmark et al. 1993; Smith et al. 1985) (Fig. 3.1). Rhizobactin was the first siderophore produced by *S. meliloti* to be structurally characterized, but production of rhizobactin 1021 seems to be more widespread among sinorhizobial strains. Rhizobactin 1021 is an asymmetrical citrate-derivative hydroxamate carrying two different acyl groups, acetyl and 2-decenoyl. Other citrate-hydroxamate siderophores are: schizokinen produced by some *Bacillus* species and cyanobacteria, aerobactin from enteric bacteria, arthrobactin from *Arthrobacter pascens*, acinetoferrin from *Acinetobacter haemolyticus,* and nannochelin A from *Nannocystis exedens* (Williams 1979; Neilands and Leong 1986; Kunze et al. 1992; Okujo et al. 1994; Wilhelm and Trick 1994; Hu and Boyer 1995). Citrate-hydroxamate siderophores represent two types of ferric ligands in one molecule, since the ferric iron is coordinated with two hydroxamates, and the 2-hydroxycarboxylate group from the citric acid.

In *S. meliloti* 1021, genes required for rhizobactin 1021 synthesis are located in the pSymA plasmid (symbiotic plasmid A) which also contains genes for nodulation (*nod* genes) and nitrogen fixation (*nif* genes). Rhizobactin biosynthesis involves at least a cluster of six genes arranged in one operon, *rhbABCDEF* (Lynch et al. 2001). A plausible biosynthetic pathway was proposed on the basis of gene disruption, predicted function of gene products, and the chemical structure of rhizobactin 1021. It was suggested that the first step involves the synthesis of 1,3-diaminopropane. This compound is produced from L-glutamic acid and L-aspartic-B-semialdehyde by a two-step reaction, catalyzed first by RhbA to produce L-2,4-diamino butyric acid and 2-ketoglutaric acid and then by RhbB, responsible for decarboxylation of L-2,4-diamino butyric acid. The 1,3-diaminopropane is then hydroxylated by RhbE to produce N^4-hydroxy-1-aminopropane which is in turn

acetylated by RhbD to form N^4-acetyl-N^4-hydroxy-1-aminopropane. The coupling of two molecules of N^4-acetyl-N^4-hydroxy-1-aminopropane to citrate may occur in a similar way as aerobactin production in *E. coli*. By analogy to aerobactin synthesis, it is proposed that this reaction consists of two steps catalyzed by a synthetase complex of two subunits encoded by RhbC and RhbF.

One step of the rhizobactin 1021 synthesis pathway requires an N-acylase for addition of the lipid moiety. RhbG is similar to some acyl-CoA-dependent acyl transferases and therefore it seems likely to be responsible for acylation with 2-decenoyl-CoA. Lynch et al. (2001) propose that acylation could be the last step of the pathway, nonetheless Challis (2005), proposes a modified biosynthetic pathway, where N^4-hydroxy-1-aminopropane could be acetylated with acetyl-CoA through RhbD or alternatively with 2-decenoyl-CoA through RhbG. Disruption of the *rhbG* gene did not affect siderophore production or uptake as determined by chrome azurol S (CAS) or bioassays, respectively. However, it could not be discounted that the nonlipidic derivative is still used as a siderophore (Lynch et al. 2001). In summary, the role of RhbG in rhizobactin 1021 biosynthesis and how the lipid moiety is incorporated, await further investigations. The secretion system used by rhizobactin 1021 is also an open question.

The outer membrane receptor for rhizobactin 1021 is encoded by the *rhtA* gene, localized upstream of the siderophore biosynthetic genes but transcribed in the opposite orientation. The RhtA amino acid sequence reveals a TonB box and is homologous with IutA, the aerobactin receptor of *E. coli* (Lynch et al. 2001).

Upstream of the *rhbABCDEF* genes and in the same operon is localized the *rhtX* gene. Interestingly, disruption of *rhtX* gene abolishes rhizobactin 1021 utilization. Moreover, RhtX (a member of the Major Facilitator Superfamily of solute transporters) alone could substitute for FhuCDB in rhizobactin 1021 transport in *E. coli*. On these bases, Cuiv et al. (2004) propose RhtX as a permease that belongs to a novel family of siderophore transport systems not related to the ABC family of transporters (Fig. 3.3).

3.2.1.3 Xenosiderophore Transport

Soil is an important niche for many diverse genera of microbes, and rhizobia must compete with these organisms for nutritional iron in the rhizosphere. One strategy employed by soil microbes for iron acquisition is to take up ferric siderophores synthesized and secreted by other organisms. These are referred to as xenosiderophores (or exogenous siderophores). In the upper layer of humic soil, streptomycetes and fungi are predominant microorganisms, and they produce the trihydroxamate siderophores desferrioxamines and desferrichrome, respectively (Winkelmann 2007). Ferrichrome and ferrioxamines can be used as iron sources by many fungi and by some Gram-positive and Gram-negative bacteria as is the case for some rhizobia strains. The use of xenosidephores was found to be a variable trait in rhizobia. It has been reported that ferrichrome and rhodotorulic acid (a trihydroxamate siderophore produced by some yeasts) stimulate the growth

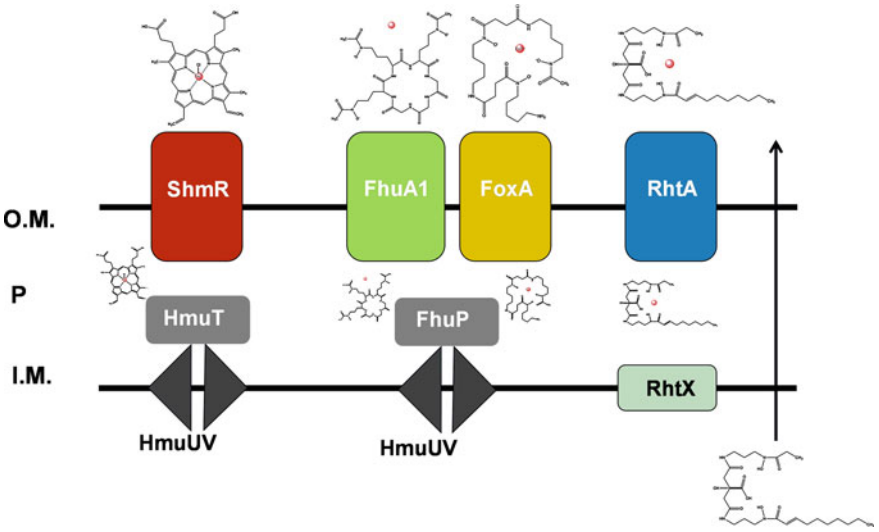


Fig. 3.3 Model of iron acquisition systems expressed in *S. meliloti*. Heme transport system involves ShmR, the outer membrane heme receptor; HmuT, a periplasmic binding protein; HmuU, a membrane permease, and HmuV, an ATPase. Ferrichrome and ferrioxamine B transport systems consist of FhuA1, the ferrichrome outer membrane receptor and FoxA, the ferrioxamine B outer membrane receptor; FhuP, a periplasmic binding protein for both siderophores and the HmuUV transporter. The ferric rhizobactin1021 transport system requires RhtA, the siderophore outer membrane receptor and RhtX, a permease that belong to a novel family of siderophore transporters. Abbreviations: *O.M.*, outer membrane; *P*, perisplasm; *I.M.*, inner membrane

of *B. japonicum* 110, LO, and 61A152 strains (Plessner et al. 1993; Small et al. 2009; Small and O'Brian 2011). Some, but not all, sinorhizobia strains tested are able to use ferrichrome, ferric rhodotorulate, and ferrioxamine B as iron sources (Smith and Neilands 1984; Reigh and O'Connell 1993). One out of eight *R. leguminosarum* strains tested can utilize ferrioxamine B, while the remaining strains could not (Skorupska et al. 1989). Only 3 out of 17 *Mesorhizobium* strains tested used ferrichrome as a sole iron source (Carlton et al. 2007). To explain the variation in the ability of using ferrichrome as iron source in *Mesorhizobium* strains, Carlton et al. (2007) suggest that ferrichrome transport systems evolved through cycles of gene acquisition and deletion, with the positive selection pressure of an iron-poor or siderophore-rich environment.

There are several reports of xenosiderophores use by rhizobia, but only a few are well characterized. Inspection of the genomes of *B. japonicum* 110, *R. leguminosarum* 3841, and *S. meliloti* 1021 reveals the presence of numerous putative TonB-dependent receptors (Fig. 3.2). In addition to endogenous siderophore receptors and putative heme receptors, the number of TonB-dependent receptors differs in these three rhizobia strains; there is only one in *R. leguminosarum* 3841, five in *S. meliloti* 1021, and eleven in *B. japonicum* 110. As only a few of them have been characterized, it cannot be assumed that they are all involved in iron

transport. In fact, two *B. japonicum* TonB-dependent receptor genes, *bll4766* and *bll6848*, have been suggested to be involved in cobalamin and nickel transport, respectively (Schauer et al. 2008). Based on growth assays, iron uptake experiments, and *in silico* analysis, no obvious receptors for catechol-like siderophore have been identified in the rhizobia, while ferrichrome seems to be the xenosiderophore more widely used.

Five putative TonB-dependent outer membrane receptor genes in *B. japonicum* strains 110 and LO are strongly induced by iron limitation (Yang et al. 2006b; Small et al. 2009b), and thus are the best candidates to encode receptors for iron siderophores. One of them, *bll4920*, is highly similar to the ferrichrome receptor *fegA* gene identified previously for *B. japonicum* strain 61A152 (LeVier et al. 1996; Benson et al. 2005), and is essential for ferrichrome utilization (Small et al. 2009b). The *fhuE* (*blr4504*) and pyoR (blr3555) genes encode outer membrane receptors for rhodotorulic acid and pyoverdine PL-8, respectively based on growth phenotypes of mutants defective in those genes (Small and O'Brian 2011).

None of the five putative outer membrane receptor genes in *B. japonicum* are proximal to other ABC transporter genes necessary for uptake into the cytoplasm as is found in *E. coli*, and thus no ferric iron transport system has been completely characterized in that species. The *fegA* gene in strain 61A152 is found in an operon with *fegB* located downstream of it (Benson et al. 2005), but the *fegB* gene is not found in strains 110 or LO (Small et al. 2009b). The function of *fegB* is unknown.

O'Connell and colleagues have revealed a novel route of ferrichrome and ferrioxamine B transport in *S. meliloti* (Cuiv et al. 2008). In *S. meliloti* 2011, a strain closely related to the sequenced *S. meliloti* 1021 strain, ferrichrome and ferrioxamine B outer membrane receptors are encoded by homologs of *smc01611* and *smc01657*. These genes were termed *fhuA1* and *foxA,* respectively, by analogy to enteric bacteria. A third *fhuA* homolog, *sma1747*, is also found (Fig. 3.2), although it is not involved in ferrichrome or ferrioxamine B uptake, and its role remains unknown. A predicted periplasmic protein encoded by the *fhuP* gene localized downstream the *foxA* gene was shown to be involved in both ferrichrome and ferrioxamine B uptake. Intriguingly, *hmuU* and *hmuV* mutants were impaired in utilization of these xenosiderophores as iron sources. The ABC system *hmuTUV* has been shown to be necessary for hemin and hemoglobin utilization in rhizobia (Fig. 3.3) (Cuiv et al. 2008).The ability of one permease complex, *hmuUV*, to transport two structurally unrelated compounds, hydroxamate siderophores and heme, is unusual. From this, it follows that more than one periplasmic binding protein should be able to interact with a single inner membrane permease, which allows the delivery of both siderophore and heme to the cytoplasm. Alternatively, iron may be released from iron compounds in the periplasm, and then transported by a common permease system. It has been proposed that in *Pseudomonas aeruginosa,* iron may be removed from some siderophores in the periplasm and that an iron-specific rather than a siderophore-specific transporter delivers the metal to the cytoplasm (Poole 2004). Whereas a ferric reductase activity may be sufficient to remove iron complexed to a siderophore, reduction of iron from heme does not release the metal, and thus a periplasmic heme-degrading enzyme would be necessary (see below). From this hmuUV likely transports two different substrates.

3.2.1.4 Ferric Iron Reduction

Once ferric chelates are taken up into cells, the iron must be reduced to the ferrous form, but the proteins responsible for this activity have only been identified in a few cases (Matzanke et al. 2004; Sedlacek et al. 2009; Wang et al. 2011). Most bacterial assimilatory ferric reductases are soluble and require flavin for activity. An exception is FhuF, an iron–sulfur cytoplasmic protein that reduces ferric ferrioxamine B (Matzanke et al. 2004).

The *B. japonicum frcB* (bll3557) gene was identified as a gene adjacent to, and co-regulated with, the *pyoR* gene encoding the receptor for ferric-pyoverdine (Small and O'Brian, 2011). FrcB is a membrane-bound, di-heme protein, characteristics of eukaryotic ferric reductases (Dancis et al. 1990; Robinson et al. 1999; McKie 2008). Heme is essential for FrcB stability, as were conserved histidine residues in the protein that likely coordinate the heme moieties. Expression of the *frcB* gene in *E. coli* conferred ferric reductase activity on those cells. Furthermore, purified, reduced FrcB was oxidized by ferric iron in vitro. *B. japonicum* cells showed inducible ferric reductase activity in iron-limited cells that was diminished in an *frcB* mutant. Steady-state levels of *frcB* mRNA was strongly induced under iron limitation in an Irr-dependent manner. FrcB belongs to a family of previously uncharacterized proteins found in many Proteobacteria and some cyanobacteria. This suggests that membrane-bound, heme-containing ferric reductase proteins are not confined to eukaryotes, but rather they may be common in bacteria.

3.2.2 Heme and Heme Proteins as Iron Sources

Heme is an iron-containing porphyrin compound essential for many cellular processes including oxygen transport, energy generation, cellular redox reactions, and metabolic regulation (O'Brian and Thony-Meyer 2002). It can also be a source of iron to bacteria that have access to it. Bacterial pathogens of animals can use hemoglobin and other heme proteins as iron sources (Wandersman and Stojiljkovic 2000; Genco and Dixon 2001; Wandersman and Delepelaire 2004). The concentration of free heme is usually very low, as it is bound tightly to hemoproteins or sequestered by serum albumin or hemopexin. Similar to siderophore transport, the use of heme or heme compounds relies on TonB-dependent outer membrane receptors able to transport heme, but not the apoprotein, into the periplasm and on ABC transporters to internalize it into the cytosol. Heme transport systems are maximally expressed when iron is scarce. Heme receptor proteins can be classified into two families. The first family comprises receptors that directly interact with free heme or hemoproteins. The second family includes receptors that depend on extracellular heme binding proteins called hemophores (Wandersman and Delepelaire 2004). Hemophores are proteins produced and secreted by the bacterium that sequester heme from diverse sources and then binds to an outer membrane hemophore receptor, followed by transport into the periplasm. Once in

the periplasm, heme is then transported into the cytoplasm by an ABC transporter. The Hem system of *Yersinia enterocolitica* (HemTUV) was among the first ABC transporters involved in heme uptake to be characterized, and is considered as a prototype for heme transport. Its homolog in *Y. pestis* is called as HmuTUV. According to the model of Hmu transport, once the heme moiety is translocated into the periplasm it is bound by a periplasmic binding protein HmuT which in turn presents it to the inner membrane permease-ATP hydrolase complex of HmuU/HmuV. Intact heme is then delivered to the bacterial cytoplasm. Interestingly, mutations in heme-specific ABC transport systems do not always result in abrogation of heme utilization by the bacterium. Recently, an alternative inner membrane system involved in heme uptake was discovered by Wandersman and co-workers (Letoffe et al. 2006, 2008), who found that the dipeptide periplasmic binding protein DppA or the L-alanyl-γ-D-glutamyl-meso diaminopimelate periplasmic binding protein MppA, are implicated in heme transport together with the dipeptide permease ABC, DppBCDF complex.

For many years, transport and degradation of heme as a mechanism to acquire iron was thought to be exclusive to pathogens. However, Noya et al. (1997) discovered that rhizobia and other non-pathogenic bacteria can use heme or hemoglobin as iron sources. Subsequently, genes involved in heme transport were identified in *B. japonicum*, *R. leguminosarum,* and *S. meliloti* (Nienaber et al. 2001; Wexler et al. 2001; Battistoni et al. 2002a; Amarelle et al. 2008). Notably, while the use of xenosiderophores was found to be variable, heme transport systems are a common trait of rhizobia strains studied so far. Moreover, rhizobia heme transport systems are present in the chromosome and not in plasmids (Fig. 3.2). As mentioned before, leghemoglobin is the most abundant protein in the cytosol of symbiotic root nodule. Although rhizobia are not in direct contact with leghemoglobin within the nodule cytosol, they may have access to it during nodule senescence. The possibility of leghemoglobin as an iron source under physiological conditions has not been addressed experimentally.

3.2.2.1 Heme Transport

The *B. japonicum bll07076* gene and the *S. meliloti smc02726* gene coding for heme receptor proteins HmuR and ShmR respectively, are required for utilization of heme, hemoglobin, and leghemoglobin. Both predicted proteins are similar to outer membrane TonB-dependent receptors, and display the typical FRAP/NPNL motif of heme receptors, although the highly conserved histidine residue in the FRAP/NPNL region is substituted by asparagine in ShmR. Despite the similarity of these proteins with other heme receptors, the identity of these two rhizobial proteins to each other is only 23 %. In *R. leguminosarum,* a predicted protein coded by *rll3522* and showing 65 % identity with ShmR was identified by in silico studies, even though its function in heme uptake has not yet been described. Analysis of the *S. meliloti* 1021 genome also reveals the presence of a second putative heme receptor protein: Smc04205. The predicted sequence of Smc04205 indicates that this protein is homologous to HasR-like outer membrane receptors

which recognize heme-bound hemophores. Moreover, the genetic region around *smc04205* is syntenous with the *hasR* regions of numerous other bacteria (Cescau et al. 2007). Nonetheless, no hemophore-like protein has yet been detected in rhizobia and no *hasR* promoter activity could be found.

In *B. japonicum*, *hmuR* is proximal and divergently transcribed from *hmuPTUV* genes. In *S. meliloti* and in *R. leguminosarum, hmuTUV* genes are distal from *shmR*. By heterologous expression in *E. coli,* Cuiv et al. (2008) confirmed that *S. meliloti hmuTUV* genes are implicated in heme transport in *E. coli*. Intriguingly, *hmuTUV* mutants in *B. japonicum*, *R. leguminosarum,* and *S. meliloti* presented a reduced but not abolished use of heme and hemoglobin as iron sources indicating that there is more than one heme transport system in those organisms (Nienaber et al. 2001; Wexler et al. 2001).

The energy for the transport of heme or siderophore-mediated iron transport across the outer membrane is provided by the TonB-ExbB-ExbD complex, where ExbBD somehow use the proton motive force to induce conformational changes in TonB. This energy coupling protein in turn interacts directly with the outer membrane TonB-dependent receptors (Postle and Larsen 2007). The precise mechanism involved is not completely understood. Moreover, it has been recently found that TonB is needed for transport of substrates in addition to iron (Schauer et al. 2008; Cornelis et al. 2009). In some organisms, there are many TonB-dependent receptors and only one TonB protein, while in other bacteria there are a few TonB-dependent receptors and more than one TonB protein (Schauer et al. 2008).

In *B. japonicum* 110, there are two clusters encoding TonB-ExbB-ExbD-like proteins, *bll7071-73* and *blr3906-08*. It is worth noting that *bll7071-bll7072* mutants are impaired in utilization of heme as an iron source, but can still use ferrichrome or ferric citrate as iron sources suggesting different functions for both TonB systems (Nienaber et al. 2001). A different situation is found in *R. leguminosarum,* where the disruption of the *tonB* homolog impedes the use of vicibactin as well as heme as iron sources (Wexler et al. 2001). In *S. meliloti* 1021 there is only one gene encoding a TonB-like protein and its function has not yet been reported.

3.2.2.2 HmuP

HmuP (*hemP*) is a small gene first identified incidentally many years ago in the sequencing of the heme transport gene cluster of *Yersinia entercolitica* (Stojiljkovic and Hantke 1992), and homologs have since been found in many Proteobacteria. However, clues into the function of *hmuP* have emerged only recently. A role for *hmuP* was identified in a screen for genes that affect expression of the outer membrane heme receptor gene *shmR* in *S. meliloti* (Amarelle et al. 2010). Expression of *shmR* in response to iron limitation is severely diminished in a *HmuP* mutant. The *shmR* gene is also controlled by RirA, but the H*muP* mutant is not defective in expression of other RirA-dependent genes. A regulatory function for H*muP* is interesting because it is found in bacteria known or suspected to have global regulators of iron homeostasis that also control heme transport. This suggests multiple levels of control of heme utilization gene expression.

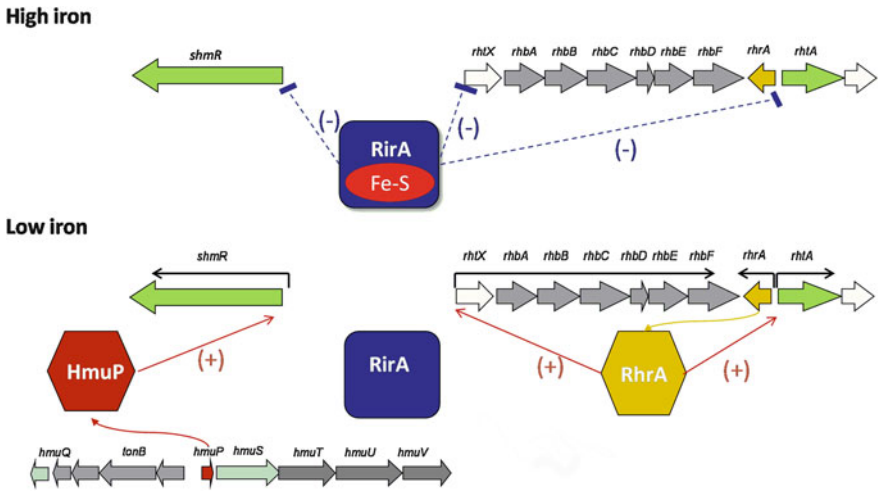


Fig. 3.4 Model of regulation of *shmR* and rhizobactin 1021 gene expression, under low and high iron conditions, in *S. meliloti* 1021. The RirA regulator may sense iron cellular status through a labile Fe atom of its Fe–S cluster, although this remains to be demonstrated experimentally. In the presence of iron, the RirA protein containing the Fe–S cluster represses expression of *shmR* gene as well as genes required for Rhizobactin 1021 biosynthesis (*rhbA*-F) and transport (*rhtA*). When cells are iron-deprived, the labile Fe is lost and the RirA regulator is not active as a repressor. Under this condition, the HmuP regulator activates the expression of *shmR* gene and RhrA activates the expression of genes required for rhizobactin 1021 biosynthesis and transport

Expression of the *S. meliloti shmR* gene under iron limitation likely involves both loss of function of the repressor RirA and the presence of an active HmuP protein (Fig. 3.4). By contrast, HmuP in *B. japonicum* functions as a co-activator with Irr to activate the HmuR operon (Escamilla-Hernandez and O'Brian 2012) (see discussion of Irr in section below). Although HmuP has not been studied in the numerous other bacteria that contain a H*muP* homolog, heme utilization genes are negatively regulated by Fur in those organisms. Thus, if HmuP plays a similar role in those bacteria as occurs in the rhizobia, thus heme utilization would likely require both derepression and activation. Further studies on the function of HmuP in the various organisms that contain different co-regulators will shed further light on this question.

3.2.2.3 Heme Degradation for Iron Release

Once exogenous heme is taken up by bacterial cells, the tetrapyrrole ring is cleaved by heme oxygenase in order to release the iron. The pioneering work on bacterial heme oxygenases was carried out with the proteins from *Corynebacterium diphtheria* (Schmitt 1997; Wilks and Schmitt 1998) and *Neisseriae meningitidis* (Zhu et al. 2000a, b; Ratliff et al. 2001; Schuller et al. 2001). These bacterial heme oxygenases have limited sequence similarity to each other and to eukaryotic heme

oxygenases, but have similar structures overall (Schuller et al. 1999; Schuller et al. 2001; Hirotsu et al. 2004). They degrade heme to iron, CO, and biliverdin.

Subsequently, a structurally unrelated heme-degrading oxygenase was described in *Staphylococcus aureus* (Skaar et al. 2004; Wu et al. 2005), and is also found in a limited number of Gram-positive bacteria, including *Bacillus anthracis* (Skaar et al. 2006). Rather than biliverdin, the oxo-bilirubin chromophore staphylobilin is a product of *S. aureus* IsdG (Reniere et al. 2010).

Because numerous rhizobia can use heme as an iron source (Noya et al. 1997), they should be able to cleave heme and release iron. The photosynthetic bacterium *Bradyrhizobium* sp. strain ORS578 cleaves heme for the synthesis of the biliverdin chromophore, and contains a gene encoding a classical HmuO protein adjacent to a phytochrome gene (Giraud et al. 2000, 2002). It is not known whether this heme oxygenase is involved in iron utilization. *Rhodopseudomonas palustris*, *Agrobacterium tumefaciens,* and *R. leguminosarum* have a similar gene arrangement in their genomes. *B. japonicum* does not have an *hmuO* gene homolog, but it does have two genes, *hmuD* and *hmuQ*, encoding weak homologs to the *S. aureus* IsdG (Puri and O'Brian 2006). *hmuQ* is within the gene cluster encoding the heme transport system identified by Nienebar et al. (2001). hmuQ binds heme with high affinity and catalyzes the cleavage of heme to biliverdin (Puri and O'Brian, 2006). Homologs of *hmuQ* and *hmuD* were identified in many bacterial genera, and the recombinant homolog from *Brucella melitensis* has heme degradation activity as well. Thus, *hmuQ* and *hmuD* encode heme oxygenases, and the IsdG family of heme-degrading monooxygenases is not restricted to Gram-positive pathogenic bacteria.

Most bacterial mutants defective in heme oxygenases have no or mild phenotypes and retain the ability to use heme as an iron source. Similarly, a *Brucella abortus* mutant defective in the gene encoding BhuQ, an IsdG family protein, grows on heme, but displays elevated levels of siderophore, indicative of iron stress (Ojeda et al. 2012). Moreover, a *bhuQ* mutant has a severe growth phenotype in a siderophore synthesis background that is rescued by $FeCl_3$ but not heme. Those workers suggest that BhuQ may make the transcriptional regulator RirA (see below) more responsive to iron-derived heme. However, it should be considered that BhuQ has a function unrelated to heme degradation.

3.2.3 Ferric Dicitrate

Citric acid can complex ferric ion and it can be used for iron transport into bacterial cells, although the affinity of iron for citrate is not as high as it is for siderophores. Some bacteria can use citrate as a xenosiderophore (e.g. *E. coli*, *Mycobacterium smegmatis, Neisseria meningitidis,* and *Pseudomonas aeruginosa*) and, occasionally, it can be used as an endogenous siderophore. For instance *B. japonicum* strains 61A152 and 110 can use citrate as siderophore, but only *B. japonicum* 61A152 strain is able to secrete it (Guerinot et al. 1990). In *E. coli* and *P. aeruginosa,* iron uptake from ferric-dicitrate is inducible by environmental citrate, and this effect requires an

extracytoplasmic function (ECF) sigma factor. The ferric-dicitrate uptake system requires the outer membrane receptor FecA, the periplasmic binding protein, and the ABC transporter system FecBCDE (Enz et al. 2000).

Although some rhizobia strains can use ferric citrate as an iron source, *B. japonicum* 110, *R. leguminosarum* 3841, and *S. meliloti* 1021 genomes have no homologs of genes encoding a FecABCDE citrate transport system. In *Streptomyces coelicolor*, exogenous citrate serves not only as an iron chelator, but also as an energy source, and thus ferric citrate is taken up for both purposes (Lensbouer et al. 2008). In this case, ferric citrate is taken up by a member of the CitMHS family of secondary transporters, a family found in numerous Gram-positive bacteria. No obvious homologs are found in sequenced rhizobial genomes.

3.2.4 Role of Iron Transport in Symbiosis

Symbiotic bacteroids have a large iron demand, but little is known about how iron gets into cells or the form of the metal that is transported. Mutations in genes encoding iron or heme transport systems described thus far do not have changed symbiotic phenotypes, indicating that such activities are not required for symbiotic function, or else they are redundant (Gill and Neilands 1989; Fabiano et al. 1995; Lynch et al. 2001; Nienaber et al. 2001; Wexler et al. 2001; Puri and O'Brian 2006; Amarelle et al. 2008; Cuiv et al. 2008; Small et al. 2009a, b). One exception is the *fegA* gene of *B. japonicum* strain 61A152, which is required for symbiosis (Benson et al. 2005). It was noted that accessibility to ferrichrome within a nodule is unlikely, so another function was suggested for this gene (Nienaber et al. 2001; Wexler et al. 2001; Benson et al. 2005). However, the corresponding gene in the USDA 122 derivative LO, *bll4920*, is not required for symbiosis, nor are the other four iron-responsive putative ferric siderophore receptor genes (Small et al. 2009a, b). Similarly, *S. meliloti* mutants defective in ferrichrome and ferrioxamine B uptake have no symbiotic phenotypes on alfalfa nodules (Cuiv et al. 2008).

The abundance of leghemoglobin in the infected plant cells of nodules makes it tempting to speculate that it is a potential iron source for bacteroids. Bacteroids are surrounded by a peribacteroid membrane to form symbiosomes, and thus the endosymbiont is physically separated from the plant globin. Heme transport across the peribacteroid membrane has not been demonstrated, and the heme transport systems employed by free-living rhizobia are not necessary for symbiosis (Nienaber et al. 2001; Wexler et al. 2001). However, leghemoglobin heme may be accessible to the bacteria during nodule senescence.

Isolated soybean symbiosomes can take up both ferric chelates and ferrous iron (Moreau et al. 1995; LeVier et al. 1996). Ferric chelate substrates are likely reduced to the ferrous form by a peribacteroid ferric reductase activity (LeVier et al. 1996), and ferrous iron can be taken up by bacteroids (Moreau et al. 1998). Soybeans express the divalent ion transporter GmDMT1 on the bacteroid membrane within nodules that may account for direct ferrous iron uptake by

symbiososmes (Kaiser et al. 2003). Thus, it is possible that ferrous iron is the form taken up by the endosymbiont.

Ferrous iron is transported by FeoB in *E. coli* and other bacteria, and homologous systems have been identified in other organisms (Hantke 2003). A putative FeoB protein encoded by *blr6523* is present in the genome of *B. japonicum* 110, but its function remains unknown. There are no homologs of the Feo system encoded on the *S. meliloti* 1021 or *R. leguminosarum* 3481 genomes. The existence of a novel ferrous iron uptake system or other divalent metal transporter involved in ferrous iron uptake requires further investigation. As noted earlier, symbiotic bacteroids may receive an adequate supply of iron from the plant host, and may not normally be iron limited. In that case, a high affinity bacterial iron transport system would appear to be unnecessary.

3.3 Regulation of Iron Homeostasis in the Rhizobia

Studies on the control of bacterial iron homeostasis have focused largely on Fur (ferric uptake regulator), a regulatory protein that responds to cellular iron. Perhaps the biggest surprise in elucidating iron metabolism in the rhizobia is that genetic regulation differs greatly from the bacterial paradigm established by *E. coli* and other model systems. Although the reductionist approach has been enormously successful, this aspect of rhizobial biology reminds us of the diversity of bacterial biology, and the need for restraint in extrapolating data from a few model systems to an entire kingdom of life. Moreover, there is substantial diversity within the rhizobia. We now know that rhizobial Fur homologs are manganese-responsive regulators, at least in some species, and that the job of iron perception and regulation is carried out by two other regulators, Irr and RirA. Furthermore, some species have only Irr, whereas others have both Irr and RirA.

3.3.1 The Fur/Mur Protein

3.3.1.1 Brief Overview of Bacterial Fur

In *E. coli* and many other bacteria, Fur represses genes involved in high affinity iron transport under iron repleted conditions, and which are derepressed when the metal is scarce. In addition, Fur is involved in numerous other facets of iron metabolism, and also in processes not obviously linked to iron, such as acid shock response (Hall and Foster 1996), synthetic pathways (Stojiljkovic et al. 1994), and the production of toxins and other virulence factors (Litwin and Calderwood 1993). Fur is the founding member of a family of regulators which also includes Zur (Gaballa and Helmann 1998; Patzer and Hantke 1998), PerR (Bsat et al. 1998),

Irr (Hamza et al. 1998; Qi et al. 1999), and Nur (Ahn et al. 2006). These proteins differ in function and have different DNA binding sites, but are all involved in metal-dependent control of gene expression.

Fur homologs are found in many bacterial genomes. Structural analysis of Fur and its DNA binding properties have been most extensively studied in *E. coli*, *P. aeruginosa* and *B. subtilis*, whereas analyses of *fur* mutants and the identification of genes under Fur control have also been studied in those bacteria and in several other organisms as well. The working model for Fur function posits that, when bound by ferrous (Fe^{2+}) iron, Fur binds its target DNA within the promoter of the regulated gene to repress transcription. However, when iron is limiting in the cell, Fur protein is unbound by iron and no longer binds DNA with high affinity, hence gene expression is derepressed.

Downregulation of Fur- and iron-responsive genes in *fur* mutants implicates positive control. In numerous cases, apparent positive control of Fur-dependent genes is an indirect effect of its repression of a small RNA that negatively regulates genes in an iron-dependent manner (Masse and Gottesman 2002; Wilderman et al. 2004; Mellin et al. 2007; Gaballa et al. 2008; Mellin et al. 2010). In *E. coli*, Fur relieves silencing of the ferritin gene by the histone-like protein H-NS by binding to multiple sites on the ferritin promoter to prevent H-NS binding (Nandal et al. 2009). Other studies indicate direct positive control by demonstrating Fur binding to target genes (Delany et al. 2004; Grifantini et al. 2004; Ernst et al. 2005; Danielli et al. 2006; Gao et al. 2008; Butcher et al. 2011; Yu and Genco 2012), although recruitment of RNA polymerase or increasing promoter strength as a consequence of Fur binding have not been demonstrated. In addition, the metal-free form of Fur from *Helicobacter pylori*can bind DNA in several promoters (Delany et al. 2001, 2003; Ernst et al. 2005).

3.3.1.2 The Fur Homolog Mur is a Manganese-Responsive Regulator in the Rhizobia and Other α-Proteobacteria

The *sitABCD* operon of *S. meliloti* was identified in a screen for mutants that could not grow in the presence of metal chelator, and was shown to be necessary for manganese acquisition (Platero et al. 2003). These genes encode an ABC system belonging to the metal transport family found in many bacteria (Claverys 2001). A *fur* gene homolog lies adjacent to the *sitABCD* genes, which led those authors to speculate that Fur may control *sitABCD*. Subsequently, three reports came out showing that this is indeed the case, but the regulatory metal is Mn^{2+}, not Fe^{2+} (Chao et al. 2004; Diaz-Mireles et al. 2004; Platero et al. 2004). Manganese-responsiveness of a *sitA* reporter fusion is lost and constitutively high in a *fur* mutant of *R. leguminosarum* or *S. meliloti*, and Fur binds to the *sitA* promoter in vitro (Chao et al. 2004; Diaz-Mireles et al. 2004; Platero et al. 2004, 2007). Furthermore, the *sitABCD* operon is not regulated by iron. Microarray analysis of an *S. meliloti fur* mutant identified 23 genes that are normally down regulated by Fur (Chao et al. 2004). Other than *sitABCD*, it is not known whether these genes

are regulated by manganese, or whether they have manganese-related functions. Mur has also been described in α-Proteobacteria other than the rhizobia. *B. abortus* Mur controls the *mntH* gene (Anderson et al. 2009; Menscher et al. 2012), and it suppressors the manganese transporter *sitABCD* operon *Agrobacterium tumefaciens* (Kitphati et al. 2007).

The *B. japonicum* Mur homolog was initially identified based on its ability to complement an *E. coli fur* mutant (Hamza et al. 1999). Mur binds the *irr* gene promoter, and a *mur* mutant shows loss of iron-responsive expression of *irr* mRNA (Hamza et al. 2000; Friedman and O'Brian 2003). In addition, global expression of numerous iron-responsive genes is aberrant in a *mur* mutant (Hamza et al. 2000; Yang et al. 2006c). Mur controls iron-dependent expression of the *fegA* gene encoding the ferrichrome receptor in *B. japonicum* strain 61A152 (Benson et al. 2004), but not in strains 110 or 122 (Small et al. 2009b). Finally, *B. japonicum* Mur is activated by either Fe^{2+} or Mn^{2+} in vitro to bind DNA and repress transcription (Friedman and O'Brian 2003, 2004). However, more recent studies show unequivocally that *B. japonicum* Mur is a manganese-responsive regulator, and its role in iron metalloregulation is probably minor or indirect in most cases.

B. japonicum Mur has three known direct targets, *irr*, *mntH,* and *mnoP*, and those genes contain a conserved motif in their promoters that binds Mur (Friedman et al. 2006; Hohle and O'Brian 2009, 2010; Hohle et al. 2011). Expression of the *mntH* and *mnoP* genes is responsive to manganese, but not iron. The apparent responsiveness of the *irr* gene to iron is now known to be due to the combined activity of Mur repression and Irr anti-repression (Hohle and O'Brian 2010). Early experiments were routinely carried out with manganese as part of the nutrient supplementation, which results in Mur occupancy of the *irr* promoter. Under iron limitation, Irr also binds to the *irr* promoter at a site that overlaps with the Mur-binding site, thus preventing Mur binding. Thus, transcript is high under iron limitation, and also in a *mur* mutant. Most other iron-regulated genes that are affected in a *mur* mutant only show modest effects, and have not been shown to be direct targets of the regulator (Yang et al. 2006c).

It is now clear that the Fur homolog is not the primary iron-responsive transcriptional regulator in the rhizobia, and that this function is taken over by Irr, RirA, or both. Thus rhizobia, and apparently many other α-proteobacteria, control iron homeostasis by a mechanism quite different from the paradigm established in *E. coli* and other model organisms.

3.3.1.3 Characterization of the Mur-binding Cis-Acting Regulatory Element

Central to the model of Fur function in *E. coli* and other model organisms is the so-called Fur box, a DNA binding element for Fur that contains similarity to a 19-bp, AT-rich palindromic consensus sequence (Fig. 3.4). Sequence similarity to a Fur box consensus within promoter regions of genes is taken as ab initio evidence for regulation by Fur. The binding site of Fur has been interpreted as two 9 bp inverted repeats, as three shorter hexameric repeats in a head-to-head-to-tail

orientation, and as two 7-1-7 inverted repeat motifs (Escolar et al. 1998; Baichoo and Helmann 2002)

B. japonicum Mur was originally identified based on its ability to complement an *E. colifur* gene mutant, thus its ability to bind to a Fur box consensus is not surprising (Hamza et al. 1999; Friedman and O'Brian 2003). Similarly, the *E. coli* Fur-regulated *bfd* gene is regulated by *R. leguminosarum* Fur/Mur in an iron-dependent manner in *E. coli* (Diaz-Mireles et al. 2004). However, these organisms do not contain an *E. coli*-like Fur box sequence. Characterization of the promoters of the Mur-regulated genes *irr, mntH,* and *mnoP* in *B. japonicum* identified the binding site as three imperfect direct repeat hexamers that are all required for normal occupancy by Fur. This site is dissimilar to the *E. coli* Fur box consensus (Friedman and O'Brian 2003; Hohle and O'Brian 2009, 2010). Although *B. japonicum* Mur binds both this element and Fur box DNA with high affinity, *E. coli* Fur does not bind to the *irr* Mur-binding site. Mur binds to the promoter as one or two dimers, and this binding is sufficient to inhibit transcription in vitro in a metal-dependent manner (Friedman and O'Brian 2003).

The *R. leguminosarum* and *S. meliloti sitA* promoters have two and one Mur-binding sites, respectively, with similar sequences (Diaz-Mireles et al. 2004; 2005; Platero et al. 2007). These cis-acting elements are described as palindromic, and although they are somewhat similar to the Fur consensus sequence, Mur binds the Fur box with lower affinity (Wexler et al. 2003; Bellini and Hemmings 2006; Platero et al. 2007). *R. leguminosarum* Mur binds each *sitA* binding site as one or two dimers (Bellini and Hemmings 2006), but *S. meliloti* Mur occupies its cognate element as a single dimer (Platero et al. 2007). The basis for these differences is unknown, but the *S. meliloti* study used much less protein in the in vitro binding analysis than the *R. leguminosarum* Mur work. Mutation of a single hexamer within the *B. japonicum irr* or *mntH* Mur-binding site can result in occupancy of only one dimer (Friedman and O'Brian 2003; Hohle and O'Brian 2009), suggesting that differences in the DNA targets may account for differential occupancy, as has been described for *E. coli* Fur (Escolar et al. 1999). A *B. japonicum* Mur mutant was identified that can only occupy the *irr* promoter as a single dimer (Friedman and O'Brian 2004), but the residues that were altered are conserved in the *S. meliloti* and *R. leguminosarum* proteins. Despite differences between the *B. japonicum* Mur-binding sites and those of *S. meliloti* and *R. leguminosarum*, computational analysis of known and putative Mur-binding sites indicates a core of common residues in all of the *cis*-acting elements (Rodionov et al. 2006). A comparison of the five known Mur-binding sites yields a similar consensus (Fig. 3.4).

3.3.1.4 Metal-Binding Properties of Mur

Mur regulators function by binding the cognate regulatory metal directly, which confers DNA-binding activity on the protein (Friedman and O'Brian 2004; Bellini and Hemmings 2006; Platero et al. 2007). A regulatory Fe^{2+}-binding site (site 1)

and a structural Zn^{2+}-binding site (site 2) implicated from the recent crystal structure of Fur from *P. aeruginosa* comprise amino acids highly conserved in many Fur family proteins, including those in the rhizobia. *B. japonicum* Mur mutants containing substitutions in site 1 or site 2 bound DNA with high affinity and repressed transcription in vitro in a metal-dependent manner. Interestingly, only a single dimer of site 2 mutant occupied the *irr* promoter, whereas the wild-type and site 1 mutant displayed one or two dimers occupancy. Both mutants were able to repress transcription from the *irr* promoter in vitro. Furthermore, both DNA binding and transcriptional repression were strictly metal-dependent. It appears that the putative functions for metal-binding sites deduced from the structure of *P. aeruginosa* Fur cannot be extrapolated to other bacterial Fur proteins as a whole.

3.3.1.5 Analyses of Mutants Suggests Novel Aspects of Manganese Uptake and Function

It is generally assumed that manganese is an essential nutrient, but recent studies on bacterial manganese transport mutants cast some doubt on whether this is generally true, and have shown differences between rhizobia and other organisms. MntH and MntABCD (SitABCD) are the two most widely represented Mn^{2+} transport systems in the eubacterial kingdom. MntH is the major high affinity Mn^{2+} transporter in numerous bacteria (Makui et al. 2000; Que and Helmann 2000; Domenech et al. 2002; Anderson et al. 2009; Hohle and O'Brian 2009), and the *mntH* gene is expressed under manganese limitation to scavenge available metal. Surprisingly, *mntH* mutants have no or mild growth phenotypes in numerous bacteria under non-stress conditions (Makui et al. 2000; Que and Helmann 2000; Domenech et al. 2002). Under normal growth, *E. coli* cells take up and contain little manganese, and manganese-dependent superoxide dismutase is not correctly metallated (Anjem et al. 2009). The lack of a manganese requirement suggests that manganese-dependent processes are not essential under those conditions, that other metals can substitute for manganese in manganese-containing proteins, or that these proteins are rendered non-essential due to compensatory activities.

B. japonicum has a single functional *mntH* gene, and no obvious *mntABC* gene homologs are present in the genome (Hohle and O'Brian 2009). A *B. japonicum mntH* mutant is almost completely defective in high affinity Mn^{2+} uptake activity. Moreover, the *mntH* strain has a severe growth phenotype under normal growth conditions (Hohle and O'Brian 2009), suggesting a greater reliance on manganese compared to *E. coli* and perhaps other organisms as well. Manganese as an essential nutrient may be a general feature of the Proteobacteria because *mntH* or *mntA* (*sitA*) mutants of *B. abortus* (Anderson et al. 2009) or *Sinorhizobium meliloti* (Platero et al. 2003; Davies and Walker 2007), respectively, also have growth phenotypes, and also a defect in manganese-dependent superoxide dismutase activity.

Nutritional metals such as manganese are available as the divalent cation in aerobic environments, and are thus soluble. Whereas cytoplasmic (inner)

membrane transporters of free metal ions are well characterized in bacteria (Patzer and Hantke 1998; Kehres et al. 2000; Tottey et al. 2001; Degen and Eitinger 2002), translocation across the outer membrane in Gram-negative bacteria into the periplasm has not been described until recently. In principle, outer membrane pores with no or low selectivity should readily accommodate the diffusion of these small, soluble nutrients that are needed only in low quantities (Nikaido 2003; Silhavy et al. 2010). However, bacteria occupy niches in which the metal is often scarce, and *B. japonicum* cells can readily take up Mn^{2+} available in the low nanomolar range (Hohle and O'Brian 2009). Thus, simple diffusion across the outer membrane down such a shallow gradient via a non-selective pore is not likely to be sufficient to satisfy the nutritional needs of the cell. In support of this idea, MnoP was identified in *B. japonicum* based on its co-regulation with the inner membrane transporter gene *mntH* (Hohle et al. 2011). MnoP is an outer membrane protein expressed specifically under manganese limitation. MnoP acts as a channel to facilitate the tranlocation of Mn^{2+}, but not Co^{2+} or Cu^{2+}, into reconstituted proteoliposomes. An *mnoP* mutant is defective in high affinity Mn^{2+} transport into cells, and has a severe growth phenotype under manganese limitation. This suggests that the outer membrane is a barrier to divalent metal ions in Gram-negative bacteria that requires a selective channel to meet the nutritional needs of the cell.

B. japonicum, *S. meliloti,* and *B.rucella abortus* all have a single manganese-dependent superoxide dismutase that is expressed under unstressed growth (Santos et al. 1999; Davies and Walker 2007; Anderson et al. 2009; Hohle and O'Brian 2012), presumably to detoxify superoxide arising from aerobic respiration. The activities of these enzymes are all diminished in a manganese transport mutant. However, a *B. japonicum mntH* mutant has a much more severe growth phenotype than does a mutant defective in the superoxide dismutase gene *sodM* (Hohle and O'Brian 2012), implying that the defective activity cannot completely explain the *mntH* phenotype.

The growth phenotype of a *B. japonicum mntH* mutant is partially rescued by replacement of glycerol with pyruvate as the carbon source (Hohle and O'Brian 2012). This raises the possibility that glycerol utilization has a manganese-dependent step that is bypassed with pyruvate. Pyruvate kinase is a glycolytic enzyme that synthesizes pyruvate from phosphoenolpyruvate, and is required for utilization of glycerol, but not pyruvate, as an energy source. Animal pyruvate kinases are Mg^{2+}-dependent enzymes, but can use various divalent metals in vitro, including Mn^{2+}. *B. japonicum* has a single pyruvate kinase, PykM, and activity of that enzyme is deficient in a *mntH* strain. Moreover, purified PykM is activated by Mn^{2+} but not by other divalent metals (Hohle and O'Brian 2012). The *E. coli* pyruvate kinase PykF was activated by Mn^{2+} or Mg^{2+}, but only maintains allosteric control by fructose 1,6 bisphosphate in the presence of Mg^{2+} (Hohle and O'Brian 2012).

Pyruvate shuttles into numerous biosynthetic and energy-generating pathways, placing pyruvate kinase, hence manganese, at a crucial metabolic intersection. In this light, it is not surprising that *B. japonicum* has not only a high affinity inner membrane transporter for Mn^{2+}, but also the specific outer membrane channel

MnoP for uptake of the metal (Hohle et al. 2011). Unlike an *mntH* strain, a *pykM sodM* double mutant has only a minor growth phenotype with pyruvate as a carbon source, implying additional manganese-dependent processes that are yet to be determined.

3.3.2 The Irr Protein

3.3.2.1 Overview of Irr

Irr is prevalent in the α subdivision of the proteobacterial phylum. Among the sequenced genomes, it is ubiquitous in the order *Rhizobiales* and *Rhodobacterales*, and found in some *Rhodospirillales* as well (reviewed in Rodionov et al. 2006). It is also present in the marine bacterium *Pelagibacter ubique*, which is in the order SAR11, but is not present in its obligate intracellular relatives *Rickettsia*, *Wolbachia,* and *Ehrlichia*. Interestingly, an Irr homolog is also found in *Acidothiobacillus ferrooxidans*, a γ-Proteobacterium that lives in acidic environments and is exposed to iron predominantly in the ferrous form. Microarray analysis shows that the vast majority of *B. japonicum* genes that are strongly regulated by iron are under the control of Irr (Yang et al. 2006b). Thus, Irr is the major iron regulator in that bacterium and probably in other *Bradyrhizobiaceae*, with Fur/Mur having a lesser or no role. Many α-proteobacteria within the *Rhizobiaceae* contain RirA in addition to Irr (Todd et al. 2002, 2005Yeoman et al. 2004; Chao et al. 2005; Viguier et al. 2005; Battisti et al. 2007; Ngok-Ngam et al. 2009; Hibbing and Fuqua 2011; Ojeda et al. 2012). The *rirA* gene is known or predicted to be controlled by Irr in numerous bacteria (Rodionov et al. 2006; Todd et al. 2006; Ojeda et al. 2012), and thus Irr may also affect the RirA regulon. Collectively, Irr and RirA appear to usurp the role of Fur in organisms that contain these novel regulators.

The *irr* gene was initially identified in *B. japonicum* in a screen for loss of control of heme biosynthesis by iron (Hamza et al. 1998), and it has been most extensively characterized in that organism. Heme is the end product of a biosynthetic pathway, culminating with the insertion of iron into a protoporphyrin ring to produce protoheme. Irr coordinates the heme biosynthetic pathway with iron availability to prevent the accumulation of toxic porphyrin precursors under iron limitation (Hamza et al. 1998). Loss of function of the *irr* gene is sufficient to uncouple the pathway from iron-dependent control, as discerned by the accumulation of protoporphyrin. This accumulation is due to derepression of *hemB* and probably *hemA* (Hamza et al. 1998; Yang et al. 2006b). Similarly, an *irr* mutant of *R. leguminosarum* has a fluorescent colony phenotype and is deregulated for the *hemA* gene (Wexler et al. 2003; Todd et al. 2006), and a *B.rucella abortus irr* mutant accumulates protoporphyrin (Martinez et al. 2005).

The Irr protein belongs to the Fur family of metalloregulators that includes Fur, PerR, Zur, Nur, and Mur (Bsat et al. 1998; Gaballa and Helmann 1998; Hamza

et al. 1998; Patzer and Hantke 1998; Chao et al. 2004; Diaz-Mireles et al. 2004; Platero et al. 2004; Ahn et al. 2006). However, Irr behaves differently from these and other regulatory proteins in fundamentally different ways, and allows novel control of iron metabolism.

3.3.2.2 Irr is a Global Regulator of Iron Homeostasis

Although Irr was initially described in the context of heme biosynthesis, it is now clear that Irr is a global regulator of iron homeostasis and metabolism (Rudolph et al. 2006; Todd et al. 2006; Yang et al. 2006b). Transcriptome analysis of *B. japonicum* shows that Irr has a large regulon, and that most genes strongly controlled by iron at the mRNA level are also regulated by Irr (Yang et al. 2006b). Numerous Irr-regulated genes contain a *cis*-acting DNA element called an iron control element (ICE) within their promoters. The ICE motif was originally identified between the divergently transcribed genes *hmuR* and *hmuT* from *B. japonicum*, and shown to be necessary for activation of those genes under iron limitation (Nienaber et al. 2001; Rudolph et al. 2006b). Irr has been subsequently shown to bind numerous ICE-containing promoters in vitro and occupy those promoters in vivo (Yang et al. 2006b; Sangwan et al. 2008; Small et al. 2006b; Hohle and O'Brian 2010; Singleton et al. 2010; Escamilla-Hernandez and O'Brian 2012). Furthermore, bioinformatic analyses identified ICE-like motifs upstream of many open reading frames in *B. japonicum* and other α-Proteobacteria (Rodionov et al. 2006; Rudolph et al. 2006b).

Transcriptome analysis in *B. japonicum* also identified many Irr-regulated genes that apparently lack an upstream ICE motif (Yang et al. 2006b), suggesting either recognition of cis-elements dissimilar to ICE or indirect control by Irr. In support of the former, Irr binds promoter DNA lacking an ICE motif in *Brucella abortus* (Martinez et al. 2006, Anderson et al. 2011) and *Bartonella quintana* (Battisti et al. 2007; Parrow et al. 2009), although ICE-like motifs are predicted in numerous other genes in those organisms (Rodionov et al. 2006).

3.3.2.3 Mechanisms of Positive and Negative Control of Target Genes by Irr

Irr is both a positive and negative regulator of gene expression, and the mechanisms of control are understood to varying extents (Fig. 3.5). As expected, numerous negatively controlled genes are known or predicted to encode proteins that contain iron or are involved in the biosynthesis of heme or iron-sulfur clusters. Numerous positively controlled genes are involved in iron transport or iron-independent proteins that have an iron-dependent isozyme. It should be noted, however, that many Irr-dependent genes identified in transcriptome analysis encode proteins of unknown function (Yang et al. 2006b).

Negative control has been studied in two *B. japonicum* genes (Rudolph et al. 2006b, Sangwan et al. 2008), one encoding bacterioferritin (*bfr*), and the other,

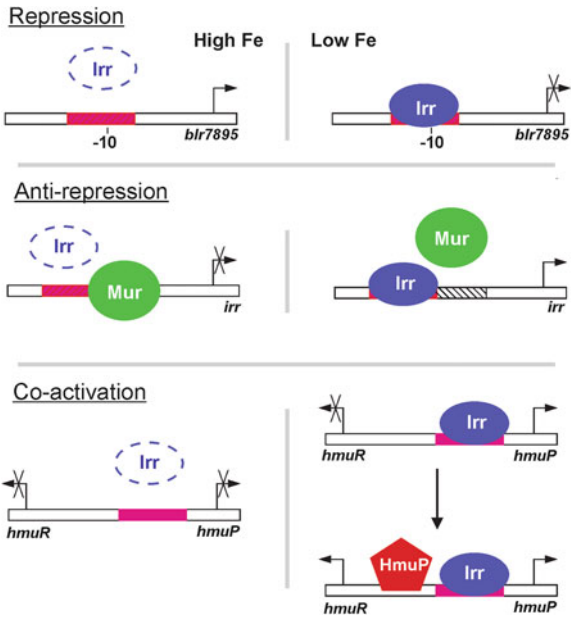


Fig. 3.5 Irr-mediated positive and negative control of iron-responsive genes. Cells grown under iron-replete conditions lack Irr activity due to degradation in *B. japonicum* and probably *B. abortus,* and by inactivation in *R. leguminosarum*. The model shown is based on *B. japonicum*, where the three types of control have been demonstrated. The shaded area of the DNA shows the Irr binding site, and the hashed area is the Mur-binding site. The bent arrow denotes the transcription start site. Although HmuP has been shown to occupy the *hmuR-hmuP* promoter in vivo, direct interaction with DNA or other proteins has not been demonstrated

blr7895, is a gene of unknown function, but a mutant in the homologous gene in *Agrobacterium tumefaciens* is sensitive to hydrogen peroxide stress (Ruangkiattikul et al. 2012). These genes are maximally expressed in the presence of iron in wild-type cells, and are derepressed in an *irr* mutant (Rudolph et al. 2006b; Sangwan et al. 2008). Correspondingly, Irr occupies the *bfr* and *blr7895* promoters under iron limitation (Sangwan et al. 2008), implicating a repressor role for the protein. Both gene promoters contain an ICE motif that overlaps the transcription site and are near or within the 10 regions (Rudolph et al. 2006a, b), and Irr binds those promoters with very high affinity in vitro (Sangwan et al. 2008). Moreover, in vitro transcription initiated from the *blr7895* promoter is inhibited by Irr (Sangwan et al. 2008). Collectively, the findings support a model of repression, whereby Irr occupancy of the target promoter is sufficient to repress expression, probably by occluding the promoter from RNA polymerase.

A case of anti-repression as a means of positive control by Irr has been described in *B. japonicum* (Hohle and O'Brian 2010) (Fig. 3.5). *Irr* mRNA levels are modestly controlled by iron, with maximal transcript found under iron limitation. The *irr* gene is occupied by Mur under manganese- and iron-replete

conditions to repress transcription. However, when iron is limited, Irr accumulates and binds its own promoter, and Mur is unbound to it regardless of the manganese status. The Mur and Irr binding sites overlap on the *irr* promoter, and Irr occupancy prevents Mur binding in vivo and in vitro (Hohle and O'Brian 2010). Moreover, Mur-dependent transcription from the *irr* promoter in vitro is relieved in the presence of Irr. Irr is not necessary for high *irr* mRNA expression in a *mur* mutant, and thus the collective evidence shows that Irr is an anti-repressor rather than an activator. This conclusion cannot be directly extrapolated to other genes positively controlled by Irr, as most of them do not appear to be regulated by Mur (Yang et al. 2006b, c; Hohle et al. 2011). Nevertheless, Irr may be an anti-repressor of other negative regulators that are yet to be elucidated.

Many gene transcripts are downregulated in an *irr* mutant compared to the parent strain in *B. japonicum* (Yang et al. 2006b), and positive control by Irr has also been implicated in *B. abortus* (Anderson et al. 2011) and *Agrobacterium tumefaciens* (Hibbing and Fuqua 2011). Studies of positively controlled genes have focused primarily on those involved in the transport of iron or heme into cells (Nienaber et al. 2001; Martinez et al. 2006; Rudolph et al. 2006b, Small et al. 2006b; Anderson et al. 2011; Small and O'Brian 2011; Escamilla-Hernandez and O'Brian 2012). In *B. japonicum*, the promoters of these genes are occupied by Irr under iron limitation in vivo (Small et al. 2006b; Escamilla-Hernandez and O'Brian 2012), and thus the control exerted by Irr is direct. In addition, promoters each contain an ICE motif that is distal from the transcription start site or −10/−35 region (Rudolph et al. 2006b; Small et al. 2006b), providing at least an inference that Irr functions by activation rather than anti-repression of those genes (Barnard et al. 2004).

Although the mechanism of activation by Irr is unknown, recent work identified HmuP as a regulator of Irr-dependent expression of the *hmuR* operon encoding proteins needed for heme utilization as a nutritional iron source (Fig. 3.5). The *hmuP* gene is found divergently from the *hmuR* operon in the context of the *hmuPTUV* operon (Escamilla-Hernandez and O'Brian 2012). A single ICE motif is found between the two divergent operons, and iron-dependent control of both operons is impaired when that motif is mutated (Nienaber et al. 2001). Moreover, Irr binds the *hmuR-hmuP* promoter region in vivo (Escamilla-Hernandez and O'Brian 2012). An *hmuP* deletion abrogates activation of the *hmuR* operon, but substantial iron-dependent control of the divergent *hmuPTUV* operon remains. HmuP binds the *hmuR-hmuP* promoter, but cannot induce the *hmuR* operon in the absence of Irr. These findings implicate HmuR as a co-activator of Irr-dependent activation of the *hmuR* operon.

3.3.2.4 Irr is Controlled Post-translationally by a Heme-dependent Mechanism

Irr functions under iron limitation, which makes it distinct from other Fur family proteins because it functions in the absence of the regulatory metal, whereas other members require direct metal binding for activity in most cases.

The Irr protein accumulates in cells under iron limitation, with very low levels in iron-replete cells in *B. japonicum* and *B.rucella abortus* (Hamza et al. 1998; Martinez et al. 2005; Anderson et al. 2011), and the mechanism controlling Irr levels have been worked out in some detail in *B. japonicum*. In that organism, Irr is a conditionally stable protein that degrades in cells exposed to iron (Qi et al. 1999). *B. japonicum* Irr contains a heme-regulatory motif (HRM) near the N-terminus that binds heme and is necessary for rapid degradation. Accordingly, Irr is stabilized in a heme-deficient background or by mutagenesis of Cys-29 within the HRM. Since the discovery of heme-dependent degradation of Irr, numerous other eukaryotic proteins have been identified that degrade in response to heme by binding to HRM motifs (Jeong et al. 2004; Ishikawa et al. 2005; Zenke-Kawasaki et al. 2007; Hu et al. 2008; Yang et al. 2008).

B. japonicum Irr fused to glutathione S transferase (GST) confers iron-dependent instability on GST, but a GST fusion containing only the N-terminal 36 amino acids of Irr, which includes the HRM, is stable (Yang et al. 2005). This means that the HRM is necessary but not sufficient for rapid degradation of Irr. In addition, a C29A mutant within the HRM eventually degrades after long-term exposure to iron, but the wild-type protein is completely stable in a heme-deficient strain. This implicates an additional heme-dependent degradation process independent of the HRM. In vitro and in vivo studies identified an instability domain that includes three consecutive histidines at positions 117-119, with His-117 and His-119 being invariant residues in Irr proteins. Mutation of the HXH domain results in a very stable protein independent of iron. This HXH domain is part of a heme-binding region distinct from the HRM. Raman and EPR spectroscopy confirm two heme binding sites, one with a cysteine coordinated axial ligand and the second with six coordinate His/His ligation (Ishikawa et al. 2011).

The activity of *R. leguminosarum* Irr is also heme-dependent, but heme does not trigger protein degradation (Singleton et al. 2010). Instead, heme binding to the conserved HXH motif decreases its affinity for target DNA. Examination of the Irr homologs reveals that those within the *Bradyrhizobiaceae, Xanthobacteraceae, Methylobacteriaceae, Rhodospirillaceae and Beijerinckiaceae* have the Cys-Pro sequence and an HRM-like domain, and the HXH motif corresponding to His-117 and His-119 of *B. japonicum* Irr are completely conserved in all of the homologs. Many Irr homologs, including that of *R.hizobium leguminosarum*, lack the HRM. This raises the question of whether Irr degradation as described for *B. japonicum* occurs in other rhizobia. Singleton et al. (Singleton et al. 2010) suggested that heme-dependent degradation of Irr may correlate with an HRM, and those that lack an HRM may result in heme-dependent inactivation rather than degradation as described for the *R. leguminosarum* Irr. This is unlikely in light of the fact that Irr accumulates only under iron limitation in *B. abortus*, which does not contain an HRM (Martinez et al. 2005; Anderson et al. 2011). In addition, the *B. japonicum* Irr derivative lacking an HRM degrades, but the rate is much slower than is found for the wild-type protein (Yang et al. 2005). *B. japonicum* has a lower affinity ferric heme binding site (Qi and O'Brian 2002) that could possibly serve a similar function as the HRM, albeit less efficiently. By analogy, Irr homologs lacking an HRM may have a compensatory mechanism that allows turnover.

3.3.2.5 Regulated Degradation of *B. japonicum* Irr Requires both Redox States of Heme

Whereas the HRM binds specifically to ferric (Fe^{3+}) heme, the histidine-rich domain binds ferrous (Fe^{2+}) heme (Yang et al. 2005). An Irr mutant in whom the three histidines are replaced by alanines is stable in vivo under iron-replete conditions (Yang et al. 2005). Irr decay follows first order kinetics (Qi et al. 1999), indicating a single mechanism for degradation. Hence the two hemes likely participate in a single degradation process rather than independent processes that occur at different rates. These findings implicate a role for the redox activity of heme in Irr degradation, and further evidence suggests that this activity leads to protein oxidation (Yang et al. 2006a). *B. japonicum* Irr degrades in response to cellular oxidative stress by a mechanism that involves heme and iron (Yang et al. 2006a). Furthermore, Irr degradation is strictly O_2-dependent in vivo (Yang et al.2006a). Irr oxidation was demonstrated in vitro, requiring heme, O_2 and a reductant. An Irr truncation that does not bind ferrous heme in vitro does not degrade in vivo. Thus, it was proposed that reactive oxygen species participate in Irr degradation not only as part of an oxidative stress response (see below), but also in normal degradation in response to iron. Protein oxidation can result in hydrolysis of peptide bonds (Berlett and Stadtman 1997) and thus, in principle, oxidation of Irr could be sufficient for degradation. However, in vivo degradation of Irr is rapid, whereas carbonylation in vitro is slow. It is probable that oxidized Irr is recognized by cellular proteases as a damaged protein that is subsequently degraded. A candidate protease has not been described thus far. Degradation of Bach1 requires the ubiquitin ligase HOIL-1, which interacts with the heme-bound form of the respective protein (Ishikawa et al. 2005; Zenke-Kawasaki et al. 2007). The ubiquitin-tagged protein is then degraded. Arginyl transferase is tagged by N-end rule ubiquitin ligases in yeast and mouse for heme-dependent degradation (Hu et al. 2008).

Redox-dependent ligand switching, although not associated with protein degradation, occurs with the transcriptional regulator CooA from *Rhodospirillum rubrum* (Roberts et al. 2004) and the redox sensor EcDos from *E. coli* (Kurokawa et al. 2004). IRP2 binds ferric heme at the HRM and ferrous heme at a conserved histidine residue (Jeong et al. 2004; Ishikawa et al. 2005), and protein oxidation is reported to be heme-dependent (Yamanaka et al. 2003). However, subsequent work strongly indicates iron-dependent degradation of IRP2 by a heme-independent mechanism that does not involve the HRM (Salahudeen et al. 2009; Vashisht et al. 2009). In those studies, IRP was shown to be degraded by an E3 ubiquitin ligase complex that includes the iron and oxygen-responsive protein FBXL5. FBXL5 contains a hemerythrin-like domain at its N-terminus that confers instability under iron or oxygen deprivation, thus the protein accumulates when those compounds are high.

3.3.2.6 *B. japonicum* Irr Interacts with Heme Locally at the Site of Heme Synthesis

A fundamental problem with heme as a signaling molecule is that it is reactive and lipophilic. Heme can catalyze the formation of reactive oxygen species, and binds non-specifically to lipids, proteins, and other macromolecules. Thus, a regulatory free heme pool is unlikely. The discovery of new and novel roles for heme as a regulatory molecule in eukaryotes and prokaryotes begs for reconciliation between these functions and the cytotoxicity of heme. This problem has been partially resolved for the Irr protein from *B. japonicum*. Ferrochelatase catalyzes the insertion of iron into protoporphyrin to form heme in the final step of the heme biosynthetic pathway. Irr interacts directly with ferrochelatase and responds to iron via the status of heme and protoporphyrin localized at the site of heme synthesis (Qi and O'Brian 2002) (Fig. 3.5). Competition of the wild-type ferrochelatase with a catalytically inactive one inhibits iron-dependent degradation of Irr even though the cell is not heme-defective. This means that Irr does not respond to a free heme pool, but rather to heme locally where it is synthesized. The dissociation binding constant (K_d) of heme for Irr is about 1 nM, which is less than one free heme molecule per cell. Irr may represent the simplest type of heme signaling mechanism, because there is no obvious need for a factor to chaperone heme from the site of synthesis to its target.

The interaction of Irr with ferrochelatase is affected by the immediate heme precursor protoporphyrin. The porphyrin-bound enzyme does not bind to Irr, which is the state of ferrochelatase when iron is limiting, and allows Irr to be active and affect the genes under its control. Thus, Irr is affected by heme and by its substrates so that heme synthesis does not exceed iron availability. In the presence of iron, ferrochelatase inactivates Irr, followed by Irr degradation to derepress the pathway. Irr is present but inactive in cells that express a catalytically inactive ferrochelatase, but active in a *hemH* deletion strain (Qi and O'Brian 2002). It is possible that inactivation of Irr allows loss of function that is faster than its degradation. Indeed, the *hemB* mRNA is elevated by iron more rapidly than Irr degrades (Chauhan et al. 1997; Qi et al. 1999).

3.3.2.7 Iron Homeostasis is Controlled by the Status of Heme via Irr

Irr interacts directly with the heme biosynthesis enzyme ferrochelatase, resulting in degradation under iron-replete conditions, or accumulation of active protein under iron limitation (Qi and O'Brian 2002). Thus, the discovery that Irr is a global regulator of iron-regulated genes indicates that iron homeostasis is controlled by the status of heme. Indeed, a heme-deficient strain of *B. japonicum* cannot maintain normal iron homeostasis. Control of Irr-regulated genes is aberrant in a heme-defective *B. japonicum* mutant, resulting in iron-replete cells behaving as if they are iron-limited (Yang et al. 2006b). The heme mutant has abnormally high cellular iron content, probably because iron transport genes are

constitutively activated due to persistence of Irr in that strain. Accordingly, under iron limitation an *irr* mutant behaves as if it were iron replete, even though cellular iron levels are lower than that found in the wild-type (Yang et al. 2006b).

Most bacteria studied to date sense and respond to iron directly to regulate gene expression. That is, iron binds directly to a regulatory protein to modulate its activity. Iron binding to Fur confers DNA-binding activity on the protein, as also occurs for the DtxR regulator from *Corynebacterium diphtheriae* and its homolog, the IdeR protein from *Mycobacterium tuberculosis* (Escolar et al. 1999; Pohl et al. 1999a, b). However, *B. japonicum*, and perhaps other α-proteobacteria, do not sense iron directly, but rather sense and respond to an iron-dependent process, namely the biosynthesis of heme. Is there an advantage to this type of control? Approximately one-half of the total iron in iron-limited *B. japonicum* cells is found in heme (unpublished observations). Since heme biosynthesis places such a high energy demand on the cell, this synthesis may serve as a sensitive indicator of the overall iron status. Also, many iron-dependent processes such as electron transport, tricarboxylic acid cycle, and detoxification are associated with aerobic metabolism, which also requires heme. Therefore, it may allow a better coordination of cellular events.

3.3.2.8 Oxidative Stress Promotes Degradation of the *B. japonicum* Irr Protein

Bacteria have multiple defense strategies against oxidative stress, including the direct detoxification of ROS by catalase, peroxidases, and superoxide dismutase. Oxidative stress responses require the activation of regulatory proteins and the induction of genes under their control. In many bacteria, the transcriptional regulator OxyR (Christman et al. 1989; Tao et al. 1989) senses hydrogen peroxide (Zheng et al. 1998) and induces numerous genes whose products are involved in peroxide defense (Tartaglia et al. 1989; Altuvia et al. 1994), redox balance (Prieto-Alamo et al. 2000; Ritz et al. 2000) and other factors (Altuvia et al. 1997; Zheng et al. 1999). In *B. subtilis*, PerR is the major peroxide regulator and represses a large PerR regulon (Herbig and Helmann 2001). The OhrR family of antioxidant regulators is responsible for organic hydroperoxide resistance (Mongkolsuk et al. 1998). *B. japonicum* contains an OxyR homolog, but it may function differently in that organism than in other systems (Panek and O'Brian 2004). Evidence points to Irr as an oxidative stress response regulator. Irr degrades in response to H_2O_2 produced endogenously in a catalase-deficient (*katG*) strain, or to H_2O_2 applied exogenously to culture media (Yang et al. 2006b). A *B. abortus irr* mutant displays elevated catalase activity and resistance to killing by H_2O_2 (Martinez et al. 2006). The Irr deficiency causes derepression of *hemB* in *B. japonicum* and elevated heme in *B. abortus*. Catalases and peroxidases are heme proteins that detoxify H_2O_2 and peroxides, respectively, and elevated *hemB* may contribute to the synthesis of those enzymes. Other examples of elevated expression of heme biosynthesis genes are noted. The *Bacillus subtilis* PerR protein mediates the induction of the

hemAXCDBL operon encoding enzymes for the early steps of heme synthesis (Chen et al. 1995; Mongkolsuk and Helmann 2002). In *E. coli* and *Salmonella*, the *hemH* gene encoding the heme biosynthetic enzyme ferrochelatase is induced in response to H_2O_2 in an OxyR-dependent manner (Zheng et al. 2001; Elgrably-Weiss et al. 2002). However, it has not been established in *B. japonicum* or any other bacterium that synthesis of catalase or peroxidase substantially increases the overall heme demand in the cell, and thus the physiological relevance of elevated heme synthesis genes is uncertain.

3.3.2.9 Irr is Involved in the Coordination of Iron and Manganese Homeostasis

Recent work indicates that the metabolism of manganese and iron are interrelated in prokaryotes and eukaryotes, and some mechanisms and rationale for the relationship is emerging. Manganese protects cells against oxidative stress, and iron has pro-oxidative properties, which provides the basis for at least some aspects of the relationship between the two metals. Manganese can substitute for iron in numerous *E. coli* enzymes, rendering those proteins less sensitive to hydrogen peroxide damage (Sobota and Imlay 2011; Anjem and Imlay 2012). This may be a general protective mechanism for mononuclear iron enzymes, which do not require the redox activity of iron for their activity. Manganese can also substitute for iron in a class Ib ribonucleotide reductase under conditions where iron is limited and manganese is available (Martin and Imlay 2011).

In *B. japonicum*, severe manganese limitation created by growth of an Mn^{2+} transport mutant in manganese limited media requires more iron for growth compared to wild-type cells (Puri et al. 2010). Correspondingly, manganese limitation results in a cellular iron deficiency. Irr is a positive regulator of iron transport, and Irr levels are attenuated under manganese limitation in wild-type cells, resulting in reduced promoter occupancy of target genes, and altered iron-dependent gene expression. Thus, manganese control of the iron status is mediate through Irr.

Irr levels remain high regardless of manganese availability in a heme-deficient mutant, indicating that manganese normally affects heme-dependent degradation of Irr. Manganese alters the secondary structure of Irr in vitro, and inhibits binding of heme to the protein. Because heme is required for Irr degradation, the data suggest that manganese stabilizes Irr by inhibiting heme binding. Thus, under manganese limitation, Irr is destabilized under low iron conditions by lowering the threshold of heme that can trigger Irr degradation.

The Mn^{2+} transporter MntH is required for growth of iron-deficient cells in *E. coli*, which has been interpreted in terms of manganese substituting for iron in activating mononuclear enzymes (Anjem et al. 2009). In that case, iron deficiency creates a need for manganese import to compensate for the lack of iron. The *B. japonicum* work also shows a requirement for *mntH* in low iron of *B. japonicum* if manganese is also deficient, but the reasons for this appear to be different from that

described in *E. coli*. In *B. japonicum*, manganese limitation causes iron limitation which can be partially rescued by increasing iron availability (Puri et al. 2010). These observations do not suggest substitution of one metal for the other, but rather a mechanism for decreasing the iron content when manganese is limiting.

It is plausible that manganese limitation renders cells more vulnerable to oxidative stress, and that attenuating the cellular iron content limits the effects of iron on oxidative stress.

The *irr* gene is transcriptionally regulated by both manganese and iron through Mur and Irr respectively, as described above (Hohle and O'Brian 2010) (Fig.3.5). Irr relieves Mur-dependent repression under iron limitation when manganese is present, and those are the conditions of maximal Irr activity. Irr binds manganese to be fully functional, and thus the anti-repression mechanism ensures maximal transcription. However, the rationale for multiple levels of control is not entirely clear in light of the fact that primary control of the *irr* gene is post-translational. One possibility is that changes in *irr* mRNA under low iron conditions increases the rate of response, but does not appreciably affect the steady-state level. Alternatively, the transcriptional control may contain an evolutionary vestige. An ancestral form of the *fur* gene may have been autoregulated in a negative manner, as has been shown in *E. coli* (Escolar et al. 1999), and *irr* arose from gene duplication of *fur*. As both Irr and Mur changed function, control by Mur was maintained but an additional anti-repressor function evolved to maintain basal mRNA level, which is necessary for post-transcriptional control.

3.3.3 The RirA Protein

The realization that Fur is not a global regulator of iron transport in *Rhizobium leguminosarum* led Johnston and colleagues to search for mutants that showed deregulation of genes that are transcriptionally controlled by iron, leading to the discovery of RirA (Todd et al. 2002). They found that numerous iron-regulated genes are constitutively high in a *rirA* mutant, indicating negative control by RirA. Similarly, RirA controls expression of the rhizobactin siderophore synthesis operon in *S. meliloti* (Viguier et al. 2005), and microarray analysis reveals a large RirA regulon that includes numerous genes involved in iron transport, energy metabolism, and exopolysaccharide production (Chao et al. 2005). Thus, some systems transcriptionally repressed by Fur in *E. coli* are negatively controlled by RirA in *S. meliloti* and *R. leguminosarum*. Although most genes downregulated by iron limitation in a microarray study are independent of RirA in *S. meliloti* (Chao et al. 2005), an analysis of the *R. leguminosarum* proteome reveals 17 proteins that are diminished in an *rirA* strain (Todd et al. 2005).

A *S. meliloti rirA* mutant has a growth phenotype in iron-replete media that is partially restored under iron limitation (Chao et al. 2005). Similarly, the growth phenotype of an *Agrobacterium tumefaciens rirA* strain is relieved by a second mutation in *irr*, a gene that functions under iron limitation (Hibbing and Fuqua

2011). The growth deficiency of the *rirA* mutants in the presence of iron may be caused by the accumulation of iron to toxic levels as a result of elevated iron transport activity. Consistent with this, the *S. meliloti rirA* strain is more sensitive to H_2O_2 in the presence of iron, presumably due to the generation of reactive oxygen species via the Fenton reaction (Chao et al. 2005). In addition, a *rirA* mutant of *A. tumefaciens* constitutively expresses iron uptake genes, and is more sensitive than the wild-type to the iron-activated antibiotic streptonigrin and to hydrogen peroxide (Ngok-Ngam et al. 2009).

As described above, the rhizobia can use heme as an iron source, which requires both its transport into cells, and cleavage of the macrocycle to release iron. A *rirA* mutant is unable to grow in the presence of heme (Chao et al. 2005). The *hemPSTU* operon is constitutively expressed in the mutant, indicating that lack of growth is due to heme toxicity rather than iron deficiency (Chao et al. 2005). This conclusion is supported by the inability to rescue the growth phenotype of the *rirA* mutant in the presence of heme by addition of inorganic iron.

RirA has not yet been studied in vitro, and thus its exact mechanism of function has not been elucidated. RirA belongs to the Rrf2 family of putative transcriptional regulators, a family that is not well characterized as a whole. The best described member is IscR, a transcriptional regulator that represses iron–sulfur cluster assembly gene operons, and activates the *suf* operon (Schwartz et al. 2001; Giel et al. 2006; Yeo et al. 2006). IscR is an iron–sulfur protein, and the three cysteines are conserved in RirA (Todd et al. 2002). IscR is an active protein both in the absence and presence of the iron–sulfur cluster. Repression of the *iscRSUA* operon requires the iron–sulfur center, but the demetallated protein activates *sufABCDFE*. The lability of the iron–sulfur cluster links its assembly to the control of O_2-regulated genes (Giel et al. 2006). Most relevant to the current discussion is that IscR responds to the iron and sulfur status (Outten et al. 2004; Gyaneshwar et al. 2005). Johnston and colleagues speculate that RirA may respond to iron through the status of an iron–sulfur cluster (Todd et al. 2006). The generality of IscR function to Rrf2 family members as a whole is not yet known. It will be important to determine whether RirA is an iron–sulfur protein, and whether the status of that moiety is sensitive to the cellular iron status.

By examining the promoters of several *R. leguminosarum* genes deregulated in an *rirA* mutant, a consensus RirA-dependent cis-acting regulatory element was identified (Yeoman et al. 2004). Deletion or mutation of these elements results in loss of iron-responsiveness. Furthermore, a bioinformatic search identified RirA-responsive elements upstream of numerous genes in *R. leguminosarum* and *S. meliloti* known to be regulated by RirA (Rodionov et al. 2006). Several iron-regulated *R. leguminosarum* genes are constitutive when introduced into *Paracoccus denitrificans* on a plasmid, but iron-responsiveness is restored when the *rirA* gene is also introduced (Yeoman et al. 2004). Collectively, it is very likely that RirA binds directly to these promoters to repress gene expression in the presence of iron.

RirA is prevalent in the *Rhizobiaceae*, which includes *Brucella*, *Bartonella,* and *Agrobacterium*, as well as *Sinorhizobium* and *Rhizobium*, but homologs are not

confined to this taxonomic group. Proteins with high similarity (at least 60 % similarity) to *R. leguminosarum* RirA are present in *Nitrobacter* (Bradyrhizobiaceae), *Labrenzia* (Rhodobacterales), and in species within the more distant γ- and β-Proteobacteria, and Firmicutes. The prevalence of RirA within the Rhizobiaceae might suggest that it is the primary iron-responsive regulator within this group. However, studies of several species do not confirm this idea according to their authors. As described above, synthesis of the *B. abortus* siderophores brucebactin and 2,3-dihydroxybenzoic acid is controlled by Irr (Martinez et al. 2005, 2006). However, a putative RirA binding site upstream of the *dhbCEBAD* operon encoding 2,3 dihydroxybenzoic acid synthesis proteins was identified in a bioinformatic study (Rodionov et al. 2006). Thus, a role for RirA in control of *B. abortus* siderophore gene expression is possible. In *B. quintana*, genes encoding heme-binding proteins (Battisti et al. 2007) and a heme utilization locus (Parrow et al. 2009) are regulated by Irr rather than by RirA. In the latter study, control of gene expression was assessed in wild type cells that overexpressed *fur*, *irr,* or *rirA* in trans. In *A. tumefaciens*, genes that are upregulated under iron limitation appear to be controlled by both Irr and RirA, whereas the *hemA* gene, which is downregulated under low iron conditions, is regulated only by Irr (Hibbing and Fuqua 2011). Finally, numerous *rirA* homologs in the *Rhizobiaceae* contain a putative Irr binding site 5' of their coding regions (Rodionov et al. 2006), and control of *R. leguminosarum rirA* by Irr has been demonstrated directly (Todd et al. 2006).

The genes encoding the regulators HmuP and RhrA are derepressed in an *rirA* mutant (Chao et al. 2005). HmuP is in turn necessary to positively control the heme receptor gene *shmR* (Amarelle et al. 2010). The *shmR* gene has a putative RirA binding site (Rodionov et al. 2006), suggesting that *shmR* expression requires direct negative and positive control by RirA and HmuP, respectively (Fig. 3.4). Similarly, the rhizobactin 1021 synthesis operon *rhtXrhbABCDEF* is positively controlled by RhrA, and a putative RirA binding site upstream of rhtX suggests direct control by RirA, in addition to indirect control through the *rhrA* gene (Lynch et al. 2001; Cuiv et al. 2004) (Fig. 3.4). DNA binding studies of RirA are sorely needed to directly address those ideas.

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