Genetic regulation of Iron Responsive Genes in *Sinorhizobium meliloti*

Thesis
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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Degree of Doctor of Philosophy, is entirely my own work and has been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work

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Acknowledgement

Une thèse ne se fait pas seule:

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Abstract

Iron is an essential nutrient for most bacteria. It is a crucial metal of many metallo-enzymes and functions in important biological systems mainly as the cofactor of redox enzymes. Bacteria must acquire iron from the environment where the metal is mainly found in the ferric iron state, which is very insoluble. In addition, they must maintain iron homeostasis. One mechanism used by bacteria for the acquisition of iron is the production of siderophores, which are low molecular weight chelators with affinity and specificity for ferric iron and which are formed and secreted under iron deplete conditions.

The regulation of iron was studied in *Sinorhizobium meliloti*, which is a free-living Gram-negative bacteria found in soil and also as an endosymbiont of *Medicago sativa* (alfalfa). A homologue of the ferric uptake protein (Fur), which regulates the uptake of iron in most Gram-negative bacteria, was identified and characterised. However, the results suggest that in *S. meliloti*, Fur does not function as an iron response regulator but actually regulates manganese uptake. Another protein, the homologue of the transcriptional iron regulator RirA in *Rhizobium leguminosarum* was identified and characterised in *S. meliloti* as the new general regulator of iron responsive genes. Results showed that RirA, under iron replete conditions, downregulates the rhizobactin 1021 siderophore biosynthesis genes and also the gene encoding the outer membrane receptor of the chelator. In addition, RirA was found to downregulate and upregulate respectively *smc02726* and *dppA1*, genes involved in haem acquisition, indicating that the regulator can function both as an activator and a repressor. Also, results showed the upregulation of *rhhG*, a putative rhizobactin 1021 siderophore gene by luteolin, a flavonoid produced by alfalfa, under iron deplete and also under iron replete conditions.
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Chapter 1:
Introduction
1.1. Introduction

Iron is an essential nutrient for all bacteria with the only known exceptions being lactobacilli and *Borellia burgdorferi* (Archibald, 1983, Posey et al., 2000). It is a crucial metal as it functions in important biological systems mainly as the cofactor of redox enzymes and it is a constituent of numerous enzymes and proteins. These include components of the respiratory chain, such as cytochromes and cytochrome oxidase, of the tricarboxylic acid cycle (aconitase, succinate dehydrogenase) and of the oxidative defense systems (catalase, peroxidase, superoxide dismutase).

However, acquiring and utilising iron can be problematic for a bacterial cell. Firstly, despite being the fourth most abundant element on earth, iron is oxidised very rapidly in the air and thus is mainly available in the environment in the ferric iron state (Fe$^{3+}$), which is very insoluble. Therefore, to acquire iron, bacteria have had to overcome its insolubility.

Secondly, even if bacteria can acquire the metal, iron has two antagonist roles in the cell. Iron can promote oxidative damage through the Fenton reaction in which iron catalyses the formation of hydroxy radicals that can damage DNA and cause mutation. On the other hand, iron can be a protector from oxidative damage, preventing it for example through the action of superoxide dismutases, which remove hydroxyl radicals and which require, iron as a cofactor.

Consequently, iron homeostasis, which is the equilibrium between uptake, intracellular utilisation and storage, is regulated in bacteria at the iron uptake level. The iron level must be carefully controlled and it must only be present in appropriate amounts to avoid any toxic effects resulting from a high concentration of the metal. An unwanted release of iron from the cellular iron handling mechanism can result in lethal reactions.
Therefore, bacteria must ensure that the level of free iron remains at extremely low levels while ensuring that there is the necessary amount of iron bound to iron storage proteins. The organism has to ensure that the iron inside the cell cannot openly interact with reactive oxygen species. Reactive oxygen species are partially reduced derivatives of molecular oxygen that are produced as a natural consequence of aerobic metabolism (Fridovich et al., 1995). The reduction products of oxygen, namely superoxide and hydrogen peroxide, could interact with iron reactions shown below producing highly reactive and extremely damaging hydroxyl radicals.

Iron reduction: \( O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2 \)  

Fenton reaction: \( Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO \)  

Haber-Weiss reaction: \( (1) + (2) \quad O_2 + H_2O_2 \rightarrow HO + OH^- + O_2 \)  

1.2. Iron acquisition systems

Bacteria have overcome the problem of iron insolubility by developing a variety of iron uptake systems. The understanding of these mechanisms has greatly improved as microbial iron acquisition has been widely studied over the last twenty years. It can be concluded that bacterial iron acquisition from the environment occurs via three main different strategies.

Bacteria have the ability to produce small molecular weight metal chelators called siderophores that can acquire ferric iron from the environment. Alternatively, bacteria can bind directly to iron transport macromolecules and acquire the ferric iron from them in a host and, finally, they are able to acquire ferrous iron from the environment through the ferrous iron transport system, termed the Feo system.
Each organism does not always have the ability to use each of these three mechanisms, but of course the more accomplished it is in iron acquisition the more diversified will be the environments it can live in

### 12.1 Iron acquisition from siderophores

The most common system by which bacteria acquire iron is the siderophore iron uptake system. Siderophores are low molecular weight chelators with affinity and specificity for ferric iron and are formed and secreted under iron deplete conditions. More than 500 siderophores have been identified so far (Drechsel et al., 1998).

The common model for iron uptake through the use of siderophores is summarized in Fig. 11. The siderophore is produced in the cytoplasm and then secreted into the environment with the assistance of specialised transport proteins. The export part of the system still remains unclear. Only in the case of enterobactin, has the export transport protein, called EntS, been characterised (Furrer et al., 2002). Then, once in the environment, the siderophore solubilises and then binds to the ferric iron. Subsequently, the metallo-protein complex binds and goes through the specialised outer membrane receptor for the siderophore. Because the siderophores (70-100 kDa) are too large to go through the porins (capacity <60 kDa) present in the membrane (Nikaido et al., 1996), under iron deplete conditions, the bacteria express receptors specific for siderophores, which are anchored in the outer membrane. These proteins are not present under iron replete conditions, to limit their use by antibiotics or bacteriophages to gain entry to the bacteria. The passage of the siderophore through the receptor proteins is achieved with the help of an energy transducing system composed of TonB, ExbB, and ExbD.

Following this, the iron-siderophore is shuttled through the periplasm to its cognate permease in the inner membrane via a periplasmic protein. It then crosses the inner membrane with the help of ABC (ATP Binding Cassette) transporters, which are composed of two identical or homogenous membrane permeases and two ATP
binding identical units present on the inner face of the membrane (Koster et al., 2001).

Once, in the cytoplasm, the ferric iron is reduced to its ferrous state ($\text{Fe}^{2+}$) by reductases (Hantke et al., 2002) and so iron is released from the siderophore due to the poor affinity between the siderophore and ferrous iron. The siderophore is then reused or degraded according to the species but there again, this part of the mechanism also still remains unclear and further investigation is needed.

Fig 1.1: Schematic of the siderophore iron uptake system in gram-negative bacteria
In 2004, a new family of transport proteins was identified in a variety of bacteria including *Sinorhizobium meliloti* and *Pseudomonas aeruginosa* (Ó Cuiv et al., 2004). The *S. meliloti* siderophore, rhizobactin 1021, is structurally similar to aerobactin, transported in *Escherichia coli* via the IutA outer membrane receptor and the FhuCDB inner membrane transport system. Ó Cuiv et al. (2004) showed that the permease RhtX could substitute for the ABC transporter FhuCDB to transport rhizobactin 1021 in *E. coli*. In addition, a homologue of RhtX termed FptX in the pathogen *P. aeruginosa* was found proximal to genes that function in iron uptake via the siderophore pyochelin and was shown to be a transporter of pyochelin. RhtX and FptX would appear to be members of a novel family of permeases that function as single subunit transporters of siderophores and are not of the ABC transporter class.

While most bacteria can produce their own siderophores in most cases they can also utilise exogenous siderophores produced by other bacteria. For example, *E. coli* can utilise as many as eight different metallo-siderophore complexes with four of them being produced by other organisms: coprogen, rhodotorulic acid, ferrioxamine, and ferrichrome. This is accomplished via the six different receptors it can produce on its outer membrane (Fig 1.2).

![Fig 1.2: Schematic representation of siderophore-mediated iron uptake systems in *E. coli* K-12.](image)

Note that the TonB-ExbB-ExbD complex energises and interacts with all the OM receptors shown (not just FepA). (Andrews et al., 2003)
12.2 Iron acquisition by bacteria in a host

Direct acquisition of iron from host proteins is the mechanism mainly used by pathogens. They have to compete with iron transport molecules in the host for the limited iron that is available. Indeed, in order to reduce the level of iron available, the host produces iron-binding proteins (lactoferrins or transferrins) in response to the pathogens' locations in the host. It also produces haem and haemoglobin binding proteins, called haemopexin and haptoglobin, which limit even more the accessibility by bacteria to iron.

12.2.1 Iron uptake from glycoproteins

The iron binding proteins transferrin, contained in human serum, and lactoferrin, contained in mucosal secretions and leukocytes, are host glycoproteins that considerably limit the concentration of iron available to invading pathogens.

Transferrin and lactoferrin receptors have been identified in bacteria such as *Neisseria* species (Cornelissen et al., 1994). Indeed, members of some families such as the Neisseriaceae can overcome the problem of iron depletion by being able to acquire transferrin-iron even if they are not capable of producing siderophores. Much of the study of the use of transferrin as an iron source has been done in *Neisseria meningitidis* due to its importance in this pathogen. The uptake of iron is achieved through the production of bi-partite receptors composed of two different proteins, TbpA and TbpB for the binding of transferrin and LbpA and LbpB for lactoferrin. Both sets of proteins are iron-regulated and are present on the outer membrane. TbpA is homologous to LbpA and TbpB to LbpB. However, some important differences in regard to physiochemical, antigenic, and immunogenic properties of the proteins in each set make them quite distinctive. For example, TbpB is a lipoprotein, which is mainly exposed on the surface of the cell. TbpB is also capable of discriminating between transferrin and Fe-transferrin. Expression of TbpB is not always necessary. TbpA demonstrates some similarities to the family of...
TonB-dependent siderophore receptors. Yet, unlike them, the bacterial transferrin receptor has to remove iron from transferrin at the cell surface. The uptake of the ferric iron is dependent on the same energy transducing system as the siderophores and the transport through the periplasm and the inner membrane is dependent on a periplasmic binding protein and ABC permease system.

2.2.2 Haem iron uptake

Many bacteria have developed outer membrane receptors for haem, the richest source of iron in mammals in order to release it in the cell. For pathogens, haem is clearly an important source of iron that can be found throughout the body at low concentrations and under different forms. Free haem is bound to hemopexin in serum while hemoglobin binds to haptoglobin. Bacteria are able to transport haem delivered as haem, haem-hemopexin, hemoglobin and finally hemoglobin-haptoglobin. The mechanism used by the bacteria to acquire these molecules is, to a certain extent, similar to the mechanism used for the uptake of siderophores.

The current mechanism proposed (Cornelissen et al., 1994) is that the glycoprotein binds to the receptor on the membrane of the bacteria. The ferric iron is removed from the iron protein and then transported through the periplasm with the help of a periplasmic protein and then crosses the cytoplasm via a membrane permease system.

A new mechanism to acquire iron from haem was recently discovered in *P. aeruginosa* (Wandersman et al., 2000) and *P. fluorescens* (Idei et al., 1999). A protein called HasA (haem acquisition system) is released by bacteria and acquires the haem bound to the haemoglobin. It then chaperones the haem to the outer membrane receptor HasR.
Under anaerobic conditions, ferrous iron can be available. To acquire it, a transport system is generated by the three genes \textit{feoABC} in \textit{E. coli} (Kammler et al., 1993). \textit{feoA} and \textit{feoC} are two genes encoding proteins with a small molecular weight below 10 kDa. Their function is still unclear but the mutation of the two genes \textit{feoA} and \textit{feoB} showed a strongly reduced ferrous iron uptake phenotype. \textit{feoB} encodes an 84-kDa cytoplasmic membrane protein with a nucleotide-binding motif situated at the N-terminus necessary for ferrous iron uptake. This indicates that ferrous iron uptake is driven by ATP hydrolysis. Also, \textit{feo} mutants were derepressed for many Fur regulated genes indicating that ferrous iron transport contributes under iron oxic conditions to the iron supply of the cells (Becker et al., 1985).
Iron acquisition by plants

Iron is an essential nutrient for plants and vital for a variety of cellular functions. Mobilisation of iron by plants is achieved by two different strategies dividing plants into two groups. Dicotyledons and non-grass monocotyledons employ reductive and proton-promoted processes reducing ferric iron to ferrous iron (Strategy I). The other group of plants, graminaceous plants (grasses) secrete plant-borne chelators or phytosiderophores (Strategy II).

Strategy I

Dicotyledons and non-grass monocotyledons reduce ferric iron before uptake (Fig 13 strategy I). The roots of dicotyledonous plants have been shown to have a short zone that can be extended under iron deplete conditions and where ferric chelates are reduced (Romheld et al., 1986). Bacterial siderophores may also serve as substrates for this reduction. The process is mediated by a plasma membrane-bound redox system. Analysis of mutants defective in ferric chelate reductase activity has proven that this step is essential for iron acquisition (Yi et al., 1996). This mechanism involves the initial reduction of ferric iron by a plasma membrane bound ferric iron-chelate reductase. Then the ferrous iron is transported through the root epidermal cell membrane.

Both the reduction of ferric iron and the transport of ferrous iron are improved under iron deplete conditions. The dicotyledons and non-grass monocotyledons acidify the rhizosphere, which is thought to occur as a result of an ATP-dependent pump that extrudes protons into the rhizosphere lowering the rhizosphere pH and so improving the solubility of ferric iron (Welkie et al., 1993).
1.2.4.2 Strategy II

Under iron deplete conditions, grasses (*Poaceae*) produce and secrete phytosiderophores. They also induce a high affinity uptake system for iron - phytosiderophores that transport the complex into the root (Ma *et al.*, 1995). This is considered to be the most efficient strategy for plant iron acquisition. So, after forming a complex with the plant iron - phytosiderophores, iron is taken up by a transporter specific for the iron - siderophore complex (Fig 1.3: strategy II.) (Römheld *et al.*, 1986). A transporter mediating the uptake of phytosiderophores has recently been identified (Curie *et al.*, 2001). Splitting of the chelate, by ligand exchange or some other mechanisms, occurs within the cell.

**Fig 1.3. Mechanisms of iron uptake by plants.**
In strategy I plants (e.g. *Arabidopsis*, pea and tomato), ferric iron chelates are reduced before the ferrous iron is transported across the plasma membrane. Strategy II plants (e.g. barley, maize and rice) release siderophores capable of solubilising external ferric iron and then transport the iron - siderophore complex into the cell. (PS: phytosiderophore) (Schmidt *et al.*, 2003).
1.3. Iron storage

Because of the toxicity of iron in the cell, bacteria had to develop a way to store iron acquired from the environment in a safe and bioavailable form within iron storage proteins (Andrews et al., 1998). Thus, iron storage proteins play a key role in iron metabolism. Their ability to sequester this element gives them the dual function of providing a storage of the metal ion and of precluding its undesirable reactivity towards oxygen, leading to the production of highly hazardous reactive oxygen species.

Three different forms of iron storage protein have been identified and characterised:

- The archetypal ferritins, also found in eukaryotes
- The haem containing bacterioferritins, only found in eubacteria
- The Dps proteins

These three categories of proteins are distantly related and so share structural and functional similarities.

The large ferritins and bacterioferritins with a molecular weight around 500 kDa can hold between 2000 and 3000 iron atoms per 24-mer while the Dps proteins, which are small with a molecular weight of 250 kDa, can only store around 500 iron atoms per 12-mer.

13.1 Ferritins and bacterioferritins

Ferritin is found in prokaryotes and eukaryotes and has been well characterised since its discovery. This holoprotein is constituted in general by 24 subunits which form the protein shell harbouring the ferric iron mineral core (Harrison et al., 1996). If the protein acquires the iron in its reduced state, it is then oxidised and stored in its ferric form. Indeed, specific sites within the ferritin molecules called the ferroxidase
centre catalyse the ferrioxidation step. These sites are located within the central regions of the individual subunits.

Mutation of the ferritin A gene (ftnA) in E. coli resulted in a ~50% reduction in stationary-phase cellular iron content following growth under iron-sufficient conditions and a reduced rate of growth under iron-restricted conditions (Abdul-Tehran et al., 1999). This suggests that the function of FtnA is to accumulate iron during post-exponential growth in the presence of excess iron for use as an intracellular iron source during subsequent growth under iron deplete conditions. No role could be discovered for FtnA in iron detoxification or redox stress resistance, although amplification of the ftnA gene reduces the sensitivity of fur (ferric uptake regulation) mutants to redox stress (Touati et al., 1995).

Ferritins are part of a large superfamily of proteins, which includes another group of iron-storage protein the members of which were identified in bacteria and which were therefore called bacterioferritins (Stiefel et al., 1979, Andrews et al., 1998). Despite their name, bacterioferritins are not restricted to bacteria. They were also found in a eukaryote (Carrano et al., 1996). Although bacterioferritins were discovered a decade before ferritins and are more widespread in bacteria than ferritins, a lot about their physiological role remains to be learned.

Bacterioferritins main striking feature is the presence of haem in the form of iron-protoporphyrin IX. There are normally 12 haem groups per 24-mer located at each of the 12 two-fold interfaces between subunits. The haem is positioned within a pocket towards the inner surface of the protein shell, with the haem being exposed to the inner cavity. However, the role of the haem remains unknown, but the presence of haem is more than likely central in distinguishing the function of the haem-free ferritins from that of the bacterioferritins.

Many bfr genes are associated with a gene (bfd) encoding a [2Fe–2S] ferrodoxin known as Bfd (the bacterioferritin-associated ferrodoxin). This protein is somewhat similar to FhuF, which is thought to be involved in intracellular reduction of
ferrichrome. The *bfr* gene is iron regulated and taken with evidence that it interacts specifically with *Bfr* and that Bfd contains a Fe-S domain, it suggests a role for Bfd in iron release from *Bfr* (Quail *et al.*, 1996, Garg *et al.*, 1996) No phenotypes are linked to the mutation of bfr in *E. coli*

### 1.3.2 Dps proteins

Another iron storage protein is the non-specific DNA-binding protein named Dps (DNA-binding proteins from starved cells) that protects DNA from cleavage caused by reactive oxygen species such as the hydroxyl radicals produced during oxidation of ferrous iron by H$_2$O$_2$ (Martinez *et al.*, 1997) It is another important component that protects against oxidative and nutritional stress. These proteins bind to DNA in stationary phase and protect it from oxidative damage (Almiron *et al.*, 1992) *E. coli* Dps was recently shown to possess iron and H$_2$O$_2$ detoxification capacity, and this novel property was proposed to act in concert with physical association with DNA to achieve its protection against oxidative hydroxy radicals (Zhao *et al.*, 2002) Indeed, work on Dps of *E. coli* has demonstrated that the protein can also store iron. It has a preference for H$_2$O$_2$ as the oxidant, with O$_2$ being rather a poor alternative. This suggests that the primary role of Dps in *E. coli* is to protect DNA against the combined action of ferrous iron and H$_2$O$_2$ in the production of the hydroxy free radical (Zhao *et al.*, 2002) Thus, Dps probably does not have a strict function in iron storage.

Redox- and iron-induced homologues of Dps were found in other bacteria and an iron-storing Dps-like protein was discovered in different bacteria including *Listeria monocytogenes* (Bozzi *et al.*, 1997) and elsewhere. Whether the Dps-like proteins from other bacteria also function mainly as DNA-protecting anti-redox agents remains to be proven.
1.4. Iron regulation

As explained before, the bacteria have to ensure that as little free iron as possible is present in the cell. Therefore, iron uptake has to be tightly regulated. Control of gene expression can be at the transcriptional and at the posttranscriptional level. A variety of general and specific regulators are employed in order that the bacteria only use the necessary and most efficient mechanisms to acquire iron.

1.4.1 Ferric uptake regulator (Fur)

1.4.1.1 Introduction

The main and most important transcriptional regulator of the iron response in gram-negative bacteria is the Ferric Uptake Regulator (Fur).

The gene encoding this protein was first discovered in 1978 through its mutation in Salmonella typhimurium that resulted in the constitutive expression of all the genes involved in the iron uptake acquisition pathways of the organism (Ernst et al., 1978). Three years later, Hantke generated the same mutation in E. coli. Mutants constitutive for the expression of beta-galactosidase were selected in an fhuA-lac fusion strains. Outer membrane receptors and the transport of siderophores were produced constitutively in such strains. They were termed fur mutants and in these fur mutant strains the synthesis of a 17-kDa protein was decreased (Hantke et al., 1981).

Subsequently, the fur gene was cloned (Hantke et al., 1984), mapped (Bagg et al., 1985), sequenced (Schaffer et al., 1985) and the protein it encodes purified (Wee et al., 1988). The fur gene, like those for most transcriptional regulators, is small encoding a 148 amino-acid protein with a 17-kDa molecular weight. The Fur protein was isolated in a single step by immobilised metal-ion affinity chromatography over zinc iminodiacetate agarose. The yield of Fur protein was determined to be approximately 130 mg for 1 litre of culture grown.
Within the bacterial genomes available now, Fur homologues have been identified in a number of cases. However, the protein has been studied in relatively few species. Structural analysis of Fur and its DNA binding properties have been most extensively studied in *E. coli* (De Lorenzo et al., 1987, 1988), *P. aeruginosa* (Prince et al., 1993) and *Bacillus subtilis* (Baichoo et al., 2002), whereas analyses of fur mutants and the identification of genes under Fur control have also been studied in several other organisms as well. Interestingly, most of these Fur homologues complement or partially complement *E. coli* Fur in an *E. coli fur* mutant.

In contrast to most of the known transcriptional regulators, Fur is a very abundant protein. Unlike LacI and Trp with respectively an estimation of 10 to 20 and 50 to 300 copies per cell (Gilbert et al., 1966, Kelley et al., 1982), the *E. coli* Fur levels determined were of 5,000 molecules during the exponential phase and 10,000 Fur molecules after oxidative stress (Hantke, 2001). Backing these results, in *Vibrio cholerae*, Fur was found at approximately 2,500 molecules during the log phase, which increases to 7,500 Fur at stationary phase (Watnick et al., 1997). The high amount of Fur could be explained by the fact that Fur tends to polymerise along the DNA. Also, it could be necessary for the large number of genes that are controlled by Fur in *E. coli*. Finally, Fur could as well play a role as a ferrous iron 'buffer' binding free ferrous iron in the cell (Andrews et al., 2003).

**14.2 Fur regulon**

To this point, as many as ninety genes have been found to be regulated by Fur (Fig 14). All the proteins in the outer membrane of *E. coli* that are derepressed in fur mutants are receptors for siderophores. From the 90 genes, as many as 60 code for the biosynthesis and transport of siderophores and about 18 are for cytoplasmic proteins involved in metabolism, proteins of iron metabolism and proteins of oxidative stress response.
In general, Fur can down regulate iron metabolism genes directly; for instance, Fur regulates the reductase *fhuF* gene in *E. coli*. The protein can also indirectly down regulate genes through its regulation of specific transcriptional regulators. For example, PchR, an AraC-like transcriptional regulator in *P. aeruginosa*, is Fur regulated. This protein, itself, up regulates *fptA*, a gene that encodes the outer membrane receptor for pyochelin (Heinrichs *et al*., 1996).

Furthermore, it was shown recently (Massé *et al*., 2003) that Fur can indirectly up regulate genes at the posttranscriptional level through its regulation of a small RNA. RyhB is Fur regulated and it functions in down regulation of genes involved in iron metabolism by binding to their messenger RNA thus inhibiting their translation. This level of regulation will be discussed in a later section.

![Diagram](Fig 1.4: Different levels of regulation by the ferric uptake regulator Fur)
Recently (Delany et al., 2004) promoters of *N. meningitidis* predicted to have Fur-binding boxes were selected for the study of the molecular interactions between Fur and the promoter regions of genes expected to play an important role in survival and pathogenesis. Interestingly, it was shown that Fur can act not only as a repressor, but also as an activator of gene expression both *in vivo* and *in vitro*. Fur bound to operators located upstream of three promoters that are positively regulated *in vivo* by Fur and iron. This experiment thus demonstrated that Fur could act as a positive transcriptional regulator.

Also, in *H. pylori*, Lee et al. (2004) investigated the global gene regulation by Fur in response to iron. Using proteome profiles, 93 protein spots were found to be up- or down-regulated more than 2-fold by either a *fur* mutation or iron-depletion. Eleven of these proteins were found to be activated by Fur, five responded to iron and the others were not iron-responsive. Seven different types of gene regulation via Fur and iron were identified. These findings demonstrate again that while the Fur protein can function as a classical transcriptional repressor, it can also function as an activator.

The investigation of *fur* homologues in the rhizobia is discussed in detail in a later section.
The accepted working model for Fur function describes how when bound to ferrous iron, Fur conformation changes and the dimer then binds the promoter region of the gene it regulates on a target DNA sequence call the ‘Fur box’ thus repressing transcription. Alternatively, when iron is limiting in the cell, bacteria use what iron they have left to ensure their supply to essential proteins and thus no iron is left to form the ferrous iron - Fur complex. The affinity of iron for Fur is quite weak and so bacterial cells can remove the iron from Fur and can thus up regulate genes required for the ‘iron deplete’ state (Fig 1.5). It is generally assumed that Fur binding blocks access of RNA polymerase to the promoter to repress transcription, but this has not been demonstrated directly.

In both the presence and absence of ferrous iron in solution, Fur appears to be a dimer (Coy et al., 1991, Michaud-Soret et al., 1997, Neilands et al., 1991) A model was suggested in which the protein has been proposed to have two domains (Coy et al., 1991, Stojilkovic et al., 1995) The C-terminal region of Fur is responsible for dimerisation and metal binding whereas the N-terminal region is involved in DNA recognition and binding.
Fig 1.5: Model of regulation by the transcriptional regulator Fur

1.4.1.4 Fur binding

The purification of Fur facilitated the investigation of its activity as a DNA binding repressor \textit{in vitro}. The ability of Fur to form a complex with iron or other metal ions and to bind upstream of the iron regulated aerobactin biosynthesis genes was shown by Baggs and Neilands (1987). Purified Fur was used to identify by footprinting the precise sequence within the promoter region bound by the regulator. In the presence of a number of divalent heavy metals (Mn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Cd$^{2+}$, and partially with Zn$^{2+}$), Fur binds primarily to a DNA sequence of 31bp within the promoter region.

Both manganese and cobalt can most efficiently replace iron. Therefore, in general, manganese is used to mimic iron (as iron oxidises in the air) during experiments. The absence of divalent metal ions decreases dramatically the DNA–binding ability
of the repressor. The sequence of the operator extends from 7 bases upstream of the –35 sequence to the bp 1 of the –10 region.

Analysis by electron and atomic force microscopes (Le cam et al., 1994) showed that the Fur-DNA complexes display a well-ordered structure indicating that protein coating is probably periodic and that the arrangement along the DNA molecule is likely helical.

In several cases, Fur-binding sites consist of two or more adjacent or overlapping 'iron boxes' suggesting the binding of several Fur dimers. To illustrate this, the aerobactin biosynthesis operon promoter ($P_{aer}$) is of particular interest. $P_{aer}$ is bound by the protein at three different sites in the promoter region depending on Fur concentrations. Fur dimers firstly bind to a high affinity site, stimulating further Fur binding at adjacent and weaker sites in a way that seems to result in Fur polymerisation along the DNA duplex. This extensive occupation of the promoter by Fur was revealed to spread over 100-bp.

DNA recognition by Fur has been controversial and is not yet conclusively understood. The interaction of the Fur protein-Fe$^{2+}$ complex with the DNA has been characterised with diverse techniques for several promoters of E. coli and other genera. These studies have revealed that every iron-dependent promoter contains a target DNA sequence with different degrees of similarity to a palindromic 5'-GATAATGATAATCATTATC-3', 19 bp consensus box. Studies in vivo confirmed that this sequence cloned downstream from a heterologous promoter is sufficient for Fur-mediated repression (Calderwood et al., 1988). Searches have yielded one promoter that matches the Fur box consensus exactly (Baichoo et al., 2002), with 14- or 15-bp matches out of 19 being more typical and 11-bp accepted as a minimum match (Ochsner et al., 1996, Tsolis et al., 1995, Baichoo et al., 2002).

Sequence similarity to a 'Fur box' consensus within promoter regions of genes is taken as ab initio evidence for regulation by Fur. However, it is necessary to explain how such a relatively small dimer interacts with such an extended operator region.
Fig 1.6: Fur binding site.
The 19-bp consensus Fur binding site for *E. coli* and various models of recognition are shown. The top sequence shows the consensus Fur binding site. The classical model shows each monomer binding a 9-bp inverted repeat (shown as arrows) of the consensus, with an A:T base pair in between. The lower right sequence depicts the hexamer model with the unit of recognition being the sequence 5'-GATAAT-3' (shown as arrows). It is uncertain how Fur would bind this sequence; some have suggested that each hexamer is recognized by a single dimer. (Lavrar *et al.*, 2003).

Firstly (as shown in Fig 1.6), it was proposed by Bagg *et al.* (1987) that Fur recognizes the sequence as a 9-bp inverted repeat separated by a single base pair. However, more recent studies from Escolar *et al.* (1998, 2000) reinterpreted the consensus as the combination of three hexameric units of the simpler model 5'-GATAAT-3' (hexamer model). The data showed that at least three adjacent hexamers were required for initial binding and that additional hexamers increased the affinity of Fur for the sequence. This is a very attractive possibility because it would permit the generation of repertoires of binding sites of varying extensions and affinities, as shown in Fig 1.7, which would allow Fur to act on some promoters as a very specific regulator and in others as a more general co-regulator.
**Fig 17 Models for Fur-DNA interactions**

A represents the overlapping-dimer binding model. In this model, each monomer (shown as an oval) binds an inverted hexamer, shown as an arrow, with two dimers required for binding the 19-bp consensus C-G base pair spacers are shown in bold. B shows the 7-1-7 model, as recently described (Baichoo et al., 2002). The arrows represent the inverted 7-mer recognized by each monomer of the dimer. The bold bases represent the base separating each 7-mer in a unit. C is an application of the overlapping-dimer binding model to an extended binding site. C-G base pair spacers are shown in bold. Numbers 1, 2, and 3 refer to dimers 1, 2, and 3 (Baichoo et al., 2002).

1415 Regulation of the Fur protein

The regulation of Fur is complex. Fur is considered to be the general iron regulator in *E. coli*. However, a special relationship exists between iron metabolism and oxidative stress. As already described, while iron is a crucial nutrient for living cells, the Fenton reaction on the other hand leads iron to form hydroxyl radicals which can be damaging to cellular components. To prevent such damage, bacteria have developed regulatory pathways to ensure that iron uptake occurs to the level necessary to fulfill the physiological requirement of the cell while limiting iron toxicity.

Touati et al. (1995) isolated *fur* deletion mutants and highlighted their sensitivity to hydrogen peroxide and the increase in mutations and oxidative damage to DNA. These results imply that Fur also plays a role in the defense against oxidative stress.
Zheng et al. (1999) showed that the regulation of Fur by OxyR and SoxRS directly reflects the chemistry between iron and reactive oxygen species.

OxyR, which senses elevated levels of hydrogen peroxide, binds to the fur promoter and induces ten-fold the expression of transcripts encoding Fur. The OxyR binding site is directly upstream of the -35 region of the promoter, which is an arrangement that has been observed at other OxyR-activated promoters.

SoxR and SoxS, on the other hand, modulate the response to superoxide-generating compounds and activate the expression of a transcript encoding both flavodoxin and Fur. Flavodoxin is encoded by the fldA gene and is located upstream of fur in the bicistronic fldA-fur operon. Flavodoxin is a flavin-containing protein involved in redox chemistry. An induction by ten-fold of the expression of the transcript is achieved by SoxS binding to the promoter region of fldA. Furthermore, SoxR activation is the result of the oxidation of the [2Fe-2S]$^{2+}$ center by superoxide. The activation of SoxR up-regulates the transcription of soxS, then, the protein SoxS activates fur.

The fur gene is also autoregulated by its own gene product in E. coli. In this case, Fur binds weakly to its own promoter via a Fur box situated in the fldA-fur intergenic region, with a binding affinity that is lower than the one for the aerobactin promoter (De Lorenzo et al., 1987). Furthermore, computational analyses have identified a 21 bp sequence closely homologous to known CAP (catabolite activator protein)-binding sites upstream of the fur promoter. Finally, MarA could also bind in the fldA-fur region. In summary, the complexity of the fur regulation suggests that Fur controls more than iron acquisition systems.

1416 Fur mutagenesis

In order to study the control of the Fur regulator, mutagenesis of fur has been undertaken. Different approaches have been taken to construct fur mutant bacterial
strains. For example, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Serratia marcescens*, *Vibrio anguillarum* and *Yersinia enterolitica* were mutated by spontaneous mutation while *E. coli*, *Shigella flexneri* and *Vibrio cholerae fur* mutants were made by insertional mutagenesis and finally *Vibrio vulnificus* was mutated by internal deletion.

Another interesting way to obtain such mutations is based on the isolation of mutants by positive selection as previously described by Silver et al. (1972), and adapted for the isolation of *fur* mutants in *E. coli* K12, *Klebsiella* and *Serratia* (Hantke, 1987). The selective medium contains a relatively low concentration of Mg$^{2+}$ but an adequate supply of Mn$^{2+}$. In the selective medium, this imbalance between Mg$^{2+}$ and Mn$^{2+}$ leads to relatively high concentrations of Mn$^{2+}$ inside the cell. It was observed that Mn$^{2+}$ represses the iron transport systems and induces a positively regulated iron-dependent gene. From these observations it seems possible that manganese directly interacts with the Fur protein, thus leading to a repression of the iron transport systems. However, an indirect mechanism is also possible where iron is mobilised in the cell by Mn$^{2+}$, thus leading to a high concentration of free ferrous iron and to a repression of the iron transport systems. Whatever the mechanism of manganese action is, the constitutive mutants were not repressed by Mn$^{2+}$ and this allowed the cells to grow, thus providing a positive selection mechanism.

Results obtained in some bacterial species suggest that Fur plays a cellular role in addition to its role in iron homeostasis. Indeed, the *fur* gene mutation appears to be lethal in *Neisseria* and *V. anguillarum*.

147 Pleiotrophic function of Fur

The Fur modulon includes several genes playing a role in iron uptake such as siderophore biosynthesis and siderophore transport as well as genes that do not play an evident role in iron uptake. Indeed, looking at Fur regulated genes, it can be noted...
that this regulator also controls functions that are not obviously related to iron metabolism. These include cellular processes as varied as the acid shock response (Hall et al., 1996), chemotaxis (Karjalainen et al., 1991) and production of toxins and other virulence factors (Litwin et al., 1993).

The growth defects of fur mutants of E. coli, P. aeruginosa, V. cholerae and Yersinia pestis suggest that fur may regulate vital functions in these organisms. E. coli and V. cholerae fur mutants have lost the ability to grow aerobically with small dicarboxylic acids as carbon sources (Hantke et al., 1987). These characteristics and potential catabolite-activator protein binding sites in the promoter region of some fur genes suggest that Fur may participate in the regulation of a broad array of genes involved in basic cellular metabolism. In some instances, Fur appears to act through and in conjunction with other regulatory proteins. Both the strain backgrounds and the type of fur mutation may affect the degree of regulatory and physiological defects.

1418 Other general iron regulators

Fur is certainly the best-known and characterised iron-responsive transcriptional regulator that acts as the general iron regulator of most gram-negative bacteria and of the gram-positive bacteria with a low GC content.

However, in 2002, a new transcriptional iron regulator was identified in R. leguminosarum (Todd et al., 2002). This protein, called RirA, rhizobial iron regulator, is responsible for the control of numerous iron responsive genes such as those involved in the biosynthesis of the siderophore vicibactin. RirA would also seem to be a general iron regulator (A. Johnson, personal communication) but more analysis has to be carried out to confirm this.

In addition, in Gram-positive bacteria with a high GC content another regulator called DtxR is responsible for iron homeostasis. This family of proteins, named after...
the diphtheria toxin repressor, is only distantly related to the gram-negative iron-dependent regulator Fur and binds a different DNA operator sequence. The diphtheria toxin repressor (DtxR) originally recognised as a repressor of the gene that encodes diphtheria toxin is now known to function as a general regulator of metabolism in gram-positive bacteria such as *Corynebacterium diphtheriae*. In this bacterium, functions down regulated by iron are production of diphtheria toxin, synthesis of the corynebactin siderophore, transport of the siderophore, and utilisation of iron from haem. Although the physiological role of DtxR in *C. diphtheriae* is similar to that of the ferric uptake regulator protein (Fur), DtxR differs from Fur in structure and cannot substitute for Fur in function. Homologues of DtxR are being detected increasingly (Feese *et al.*, 2001).

In mycobacteria, *Mycobacterium tuberculosis* contains as many as four such iron-dependent regulators. IdeR is the only protein for which experimental evidence of a role in iron binding and DNA binding exists (Schmitt *et al.*, 1995). It contains extensive similarity to the DtxR family. In addition, *M. tuberculosis* contains two genes, *furA* and *furB*, that encode proteins more similar to *E. coli* Fur. Finally, there is SirR, putatively described as an iron-dependent regulator based on similarity to SirR from *Staphylococcus epidermidis* (Hill *et al.*, 1998). The iron-responsive regulatory protein encoded by *ideR*, homologue of the *dtxR* gene from *C. diphtheriae*, is the best characterised protein. It has been functionally characterised both *in vitro* and *in vivo*. The role of IdeR in the repression of siderophore production was shown with the construction of an *ideR* mutant of *Mycobacterium smegmatis* (Dussurget *et al.*, 1996). This mutant produces siderophore when grown in high- or low-iron media, demonstrating the requirement for IdeR to repress siderophore production under high-iron conditions. However, not surprisingly in light of the presence of *furA*, *furB*, and *sirR* in *M. smegmatis*, the mutant was still capable of upregulating siderophore production under low-iron conditions, suggesting the presence of a second iron-sensing regulator in *M. smegmatis*. 

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14.2 Specific transcriptional regulation

In iron acquisition systems, three main categories of positive transcriptional regulators have been identified so far. There are the alternative sigma factors, classical two component sensory transduction systems and, finally, AraC-like proteins. These systems function in a more specific way than Fur. The AraC-like mechanism of regulation is of particular interest in regard to the iron response in *S. meliloti*.

14.2.1 AraC-like transcriptional regulators

AraC transcriptional regulators are called so, based on homology to a 99 amino acid sequence of the first member of this family discovered, AraC from *E. coli* which regulates arabinose (Sheppard *et al.*, 1967). The 99 amino acids motif is found commonly in the C-terminal, though it can sometimes be located at the N-terminal (CafR and Rob from *E. coli*) or in the central domain (Ada from *E. coli* and *S. typhimurium*). These regulators of the AraC family are usually small, like most transcriptional regulators, with a size between 250 to 300 residues long.

The first member of the AraC family was identified in 1966 by Sheppard *et al.* in *E. coli*. Most of the members of this family are positive transcriptional regulators with so far two exceptions, the AraC protein from *E. coli* which can act both as a repressor and as a positive regulator (Tobin *et al.*, 1987, Shleif *et al.*, 1992) on different promoters or on the same promoter depending on the presence or the absence of appropriate effectors and YbtA from *Y. pestis*, which has an uncertain mode of action (Fetherston *et al.*, 1996). AraC-like regulators have their DNA binding domain predicted to be organised as a helix turn helix motif located on the C-terminal. In general, transcription of the regulatory gene is divergent from the gene(s) they regulate.
These proteins are involved in the regulation of three main functions

♦ Carbon metabolism controlling the degradation of sugars with for instance, AraC for arabinose or MelR and MsmR for melibiose

♦ Some AraC-like regulators control genes that are involved in pathogenesis. They may be required for the stimulation of the synthesis of proteins playing a role in adhesion to epithelial tissues, such as fimbriae, components of the cell capsule, and mvasins. Some members of this family control the production of other virulence factors such as siderophores.

♦ Finally, some regulators function in the response to stressors, such as oxidative stress (SoxS from E. coli and S. typhimurium) (Amabile-Cuevas et al., 1991, Wu et al., 1991)
1.4.2.2 The AraC protein:

AraC, a DNA-binding protein is a transcriptional regulator controlling the expression of the genes in the arabinose operon. *E. coli* can grow and utilise arabinose as its sole source of carbon and energy. The enzyme activity necessary to convert arabinose into a component of the pentose phosphate shunt is significant and the levels of the enzymes have to be regulated.

Four transcriptional units are involved in the utilisation of L-arabinose (Fig 1.8):

- *araBAD* (Englesberg et al., 1962) which encode three enzymes involved in the catabolism of L-arabinose

- *araE* and *araFGH* encoding proteins involved in the transport of L-arabinose (Brown et al., 1972; Stoner et al., 1983).

- *araC*, encoding the regulator AraC that transcriptionally controls these genes and autoregulates its own synthesis (Lee et al., 1981).

![Organisation of the genes of the L-arabinose operon](image-url)
1423 The DNA looping phenomenon

As shown in Fig 1.9, in the absence of arabinose, the AraC dimer binds the two half sites of the DNA called I₁ and O₂ separated by 210 bp, one monomer of the AraC dimer for each half site. That way, the formation of a loop occurs and prevents the transcription from $P_{araBAD}$ and from $P_{araC}$. The loop interferes with the access of RNA polymerase to the two promoters in the looping region and also stops the DNA-binding domain of AraC binding to $I₂$.

However, in the presence of arabinose, a conformational change of the protein occurs and instead of forming a loop, AraC binds to the adjacent half sites I₁ and I₂ on the DNA so that transcription from $P_{araC}$ and $P_{araBAD}$ is promoted through direct interactions of AraC with the RNA polymerase.

The $ara$ promoters are also regulated at the transcription level by the catabolite activator protein CAP which stimulates the transcription from the $araBAD$ promoter in an AraC dependent manner. Part of this stimulation is due to CAP breaking the repression loop generated between O₂ and I₁ and part is independent of looping.

1424 The light switch mechanism

AraC is a homodimer, which mainly forms dimers in solution. To operate, the AraC protein possesses two distinct domains that function independently in protein chimeras and that are connected by a flexible linker. The N-terminus of the protein permits AraC to form dimers and this is the domain that binds to the inducer, L-arabinose. The C-terminal domain is the domain that binds to the promoter regions of the genes it regulates.

Another interesting feature of AraC is its light switch mechanism (Fig 1.9). The mechanism of action of the protein is dependent on the presence or absence of the inducer. Following crystallography of AraC in the presence or absence of arabinose
(Soisson et al., 1997), Schleif (2003) proposed a mechanism for the regulator explaining the effect arabinose could have on the protein shift from looping to binding to the close half sites \( I_1 \) and \( I_2 \). This mechanism is based on the difference in the structure of AraC depending on the presence of arabinose. When the inducer is absent, the N-terminal arms of the protein bind the C-terminal DNA binding domains to hold them in a state where the protein prefers the loop. However, in the presence of arabinose, the arms are pulled off the C-terminal domains inducing them to bind to the adjacent \( I_1 \) and \( I_2 \) and initiating transcription (Saviola et al., 1998).

![Fig 1.9: Light switch mechanism of the AraC protein in E. coli](image)

Binding of AraC in \textit{trans} to the \( O_2 \) and \( I_1 \) half-sites to form a DNA loop in the absence of arabinose and its binding \textit{cis} to the \( I_1 \) and \( I_2 \) half sites in the presence of arabinose that leads to unlooping and induction of \( p_{BAD} \) and transient derepression of \( p_C \) and the light-switch mechanism (Schleif \textit{et al.}, 2003)

AraC can strongly activate transcription only when the promoter-proximal half site is overlapping the \(-35\) region of a promoter. Furthermore, it has been shown that the position of the promoter distal half site is important in order to achieve an optimal activation by AraC (Reeder \textit{et al.}, 1993).
1425 AraC-like iron regulators

So far, four examples of AraC-like regulators with a role in iron regulation have been published. One of those is RhrA (Lynch et al., 2001) described in this thesis while the other three proteins are involved in siderophore production and transport in important gram-negative pathogens.

*AlcR in Bordetella pertussis and Bordetella bronchiseptica*

The first of these AraC-like transcriptional regulators is AlcR, which is found in both *B. pertussis* and *B. bronchiseptica*. Both species are pathogens that inhabit the respiratory mucosae of humans and non-human mammals. Under iron deplete conditions, they produce a siderophore called alcaligin.

The biosynthesis genes of alcaligin are in the *alcABCDE* operon and its outer membrane receptor is encoded by *fauA*. In 1998, Beaumont and Pradel (1998) identified and characterised AlcR. This protein was found to regulate the biosynthesis of the siderophore and of its outer membrane receptor. The gene encoding the regulator is located downstream from the biosynthesis genes and is part of the *alcABCDE* operon.

The iron starvation stress response is regulated at the transcriptional level by the metallo-Fur complex and therefore, as with most siderophores in gram-negative bacteria, alcaligin biosynthesis and its receptor are down regulated by Fur under iron replete conditions.

In addition to being iron and Fur regulated, the *alc* operon was also shown to be alcaligin and AlcR dependent. Brickman et al. (2002) have shown that the activation of the transcription of the *alc* operon by AlcR can occur at extremely low concentrations of alcaligin inducer. So, the siderophore is a vital participant along
with AlcR in a positive autogenous control circuit regulating its own production and transport.

AlcR expression is itself down regulated mainly by Fur acting at the \textit{alcABCDER} operon but also at the secondary promoter-operator in the \textit{alcR} upstream region. However, there is no evidence so far for negative auto regulation of AlcR.

\textbf{\textit{YbtA in Y. pestis}}

The second example of an AraC transcriptional regulator is YbtA in \textit{Yersinia pestis}, the causative agent of plague. Under iron deplete conditions, \textit{Y. pestis} produce a siderophore called yersiniabactin or yersiniaphore (Fetherston \textit{et al}, 1995, Wake \textit{et al}, 1975) This siderophore is also produced by \textit{Y. enterola\textit{t}tica} and for which, confusingly, a separate nomenclature for homologous genes has been used in the literature.

The biosynthesis genes of the siderophore have not yet been identified with certainty but it was determined that \textit{irp2}, which encodes a 190 kDa iron regulated high molecular weight protein called HMW2 found in \textit{yersinia} species is involved (Carmel \textit{et al}, 1989, Fetherston \textit{et al}, 1995, Guilvout \textit{et al}, 1993) This gene is part of what is more than likely the yersiniabactin biosynthesis operon (Carmel \textit{et al} 1992, Fetherston \textit{et al}, 1995) \textit{psn} encodes the outer membrane receptor for both pesticin and yersiniabactin.

YbtA controls the expression of the siderophore yersiniabactin biosynthesis protein encoded by \textit{irp2} and the expression of its outer membrane protein encoded by \textit{psn}. Expression of these genes is Fur and iron regulated but the full induction also requires YbtA and probably also its siderophore, as the mutation of \textit{irp2} decreases the expression of \textit{psn}. It could be that the siderophore acts as a positive signal molecule and directly binds to YbtA to activate transcription.
Fetherston et al. (1996) identified inverted repeats in the psn promoter region, which were putative candidates for YbtA binding sites. These repeats are located 48 and 68 bp upstream from the transcriptional start site (Rakin et al., 1994) and the promoter-proximal repeat overlaps the –35 region. Mutation of the promoter-distal repeat in psn led to a decrease in but not a total loss of promoter activity.

In addition to the regulation of the outer membrane receptor, YbtA also regulates the biosynthesis genes of yermiabactin. A ybtA mutation resulted in reduced expression of the receptor and of the putative biosynthetic genes. Furthermore, a sequence nearly identical to the repeats found in the psn promoter region were also identified in the promoter region of the rrp2 operon.

Finally, YbtA is a negative regulator of its own expression. Interestingly, there are two sequences resembling a putative YbtA-binding half site located downstream of the –10 region of the ybtA promoter. It is possible that activation versus repression of YbtA may be determined by the location of its putative binding sites (–10 versus –35 regions).

**PchR in P. aeruginosa**

The third example of an AraC-like regulator of iron responsive genes that has been investigated to date is PchR in *P. aeruginosa*. This organism is a versatile Gram-negative bacterium that is found ubiquitously. Patients with cystic fibrosis, burn victims, individuals with cancer, and patients requiring extensive stays in intensive care units are particularly at risk of disease resulting from *P. aeruginosa* infection. This bacterium produces two siderophores under iron deplete conditions, pyoverdin and pyochelin. Both have been shown to contribute to the virulence of the pathogen (Cox et al., 1982).

The outer membrane receptor for pyochelin is encoded by *fptA*. The expression of *fptA* is Fur regulated (Ochsner et al., 1996) as is the biosynthesis of pyochelin.
through the regulation of the two operons *pchDCBA* (*Sermo et al., 1997*) and *pchEFGHI* (*Reimmann et al., 1998*). Those genes are also positively regulated by PchR, an AraC-type regulatory protein encoded by *pchR*, which is itself Fur regulated (*Heinrichs et al., 1996, Ochsner et al., 1996*).

The positive regulation of *fptA* through PchR was shown through the mutation of *pchR* and this is also dependent on the presence of pyochelin.

Two partially conserved heptameric repeats were identified upstream of *fptA* in the −35 region and could be putative binding sites for PchR. The same repeats sites were also identified upstream of *pchR* suggesting that PchR is likely to bind to these repeats.

**Comparison of AraC-like iron response regulators in pathogenic bacteria**

*B. pertusis*, *Y. pestis* and *P. aeruginosa* have numerous striking similarities in relation to their AraC-type iron regulators but also some differences. The three systems are Fur regulated with similar siderophores, outer membrane receptors and regulators. The three AraC-like regulators positively activate the expression of the siderophore biosynthesis genes and of the outer membrane receptors under iron deplete conditions, activation being reported to be siderophore dependent in the three cases.

The involvement of the siderophore is not well understood. Some suggested that the molecule would bind directly to the transcriptional regulator. This is however unlikely. Brickman et al. (2002) have another theory. Their study on AlcR resulted in the loss of the inducer requirement suggesting that the natural level of AlcR expression is a determinant for the controlled induction of AlcR-mediated transcriptional activation by the siderophore. They hypothesise that in the case of *B. bronchiseptica*, an inactive AlcR protein conformation exists in equilibrium with an active AlcR conformation that is competent for transcriptional initiation. The
postulated role of the siderophore would be to shift that equilibrium toward the active AlcR conformation by binding to the inactive regulator protein. Overproduction of the regulator protein would also be predicted to increase the concentration of the active conformation, thus suppressing the requirement for the inducer. This is not the first time that this observation of siderophore-dependent expression of siderophore receptor synthesis was observed (Gensberg et al., 1992).

In P aeruginosa, the bacterium devotes its energy to synthesising the molecules for the most efficient iron uptake system in a given environment. So, in a particular situation, the siderophore that is the most successful in chelating iron when coming back into the cell will upregulate the transcription of the genes related to the adequate production and transport of the siderophore.

An important difference between YbtA and PchR is that in Y pestis, mutations in siderophore production do not affect expression from the psn gene promoter and YbtA does not appear to be converted from an activator to a repressor in the absence of siderophore (Gensburg et al., 1992, Heinrichs and Poole, 1996, Fetherston et al., 1996).

Finally, in the case of YbtA and PchR, the regulator is also able to negatively autoregulate itself. So far, this feature did not appear significantly in the investigation of AlcR. This negative regulation would allow the cell to maintain a somewhat constant and low level of activator in order to retain the capacity to control the target genes (Heinrichs et al., 1996).
14.3 Post transcriptional regulation of iron responsive genes

Most literature regarding bacterial iron acquisition suggests that it is controlled mainly at the transcriptional level by the general well-characterised Fur protein. Indeed, until recently, it was thought that posttranscriptional regulation was limited to a small number of genes, but this view is changing.

It is now clear that posttranscriptional control of gene expression, including genes involved in iron acquisition in bacteria, is more important than originally thought. Identification and characterisation of new global post transcriptional regulators along with a better understanding of the mechanisms of sRNAs (small RNAs) have led to the identification of a high number of genes subject to post transcriptional regulation. This has helped to elucidate some gene control mysteries such as the one regarding the positive regulation of members of the Fur regulon by the Fur repressor in E. coli.

14.3.1 The Hfq protein

A major regulator involved in posttranscriptional regulation is the Hfq protein. The Hfq regulator, also called HF-1 (Host Factor I) was first identified in 1968 as a host factor required for the replication of Q-Beta RNA bacteriophage (Franze de Fernandez, 1968). This thermostable protein with a molecular weight of 11.2 kD is encoded by the hfq gene situated at 94.8 min on the E. coli chromosomal map (Kajitani et al., 1994, Blattner et al., 1993). The different Hfq homologues identified in bacteria show that the protein is strikingly conserved and is an abundant protein that is found primarily in the cytoplasm with the ribosomes at a copy number between 30,000 and 60,000. It works by binding strongly to single-stranded RNAs that are rich in As and Us (Moller et al., 2002, Zhang et al., 2002).
The importance of Hfq was highlighted by the disruption of its gene, which affects the expression of many genes, activating or repressing the activity of over 50 proteins. Its mutation causes pronounced pleiotropic effects including decreased growth rates and yields, decreased negative supercoiling of plasmids in stationary phase, increased cell size, osmosensitivity, oxidation of carbon sources, and sensitivity to ultraviolet light (Tsui et al., 1994, Muffler et al., 1997).

Also, it was recently discovered that a homologue of *E. coli* Hfq in *P. aeruginosa* can functionally complement Hfq in an *E. coli hfq* mutant (Sonnleitner, 2002).

Nearly four decades after its discovery, it is now established that Hfq is an RNA binding protein required for the degradation of some RNA transcripts and the efficient translation of others (Kajitani et al., 1994, Azam et al., 2000). Hfq targets several mRNAs for degradation by binding to poly(A) regions and stimulating poly(A) adenylation (Hajnsdorf and Regmer, 2000). It also represses mRNA translation by preventing ribosome binding as observed for *ompA* mRNA (Vytvytska et al., 2000). Furthermore, Hfq has been shown to interact with several small-untranslated regulatory molecules also called riboregulators, for instance, OxyS, DsrA, Rpra and Spot42, and is required for RNA regulation of the sigma S gene by OxyS, DsrA and RprA (Zhang et al., 1998, Majdalani et al., 2001, Wassarman et al., 2001).

**14.3.2 Indirect regulation by the binding of Hfq to sRNA**

Exposure to hydrogen peroxide can induce the synthesis of the sRNA OxyS, a general regulator that activates and represses the expression of multiple genes and acts also as an antimitator that protects cells against DNA damage (Altuvia et al., 1998, Zhang et al., 1998). OxyS RNA repression of *fhlA* is achieved through two base pairing interactions (Altuvia et al., 1998, Argaman et al., 2000). One site overlaps the ribosome-binding site and a second site resides within the coding sequence of the *fhlA* RNA. The OxyS RNA-*fhlA* mRNA base pairing prevents
ribosome binding and thus represses translation (Fig 1.10). The mechanism of OxyS RNA repression is less clear, but has been shown to require the RNA binding protein Hfq (Zhang et al., 1998).

**Translation Repression**

![Diagram of Translation Repression](image)

**Fig 1.10: Translation repression model with sRNAs**

DsrA is a sRNA that regulates the translation of two global regulatory proteins in *E. coli*. DsrA activates the translation of RpoS while repressing the translation of H-NS in the same way that OxyS regulates the translation of *fhlA*. At low temperature, DsrA increases the translation of RpoS by binding to the complementary sequence in the 5'-untranslated region of the *rpoS* mRNA (Lease et al., 1998; Majdalani et al., 2001; Brescia et al., 2003). This binding leads to the formation of an alternative secondary structure in the *rpoS* mRNA that is translationally active (Fig 1.11).

**Translation Activation**

![Diagram of Translation Activation](image)

**Fig 1.11: Translation activation model with sRNAs**
Small RNAs have also been shown to function in the regulation of the iron response. In particular, RyhB plays a role in the response of *E. coli* to iron stress, where it promotes the degradation of target transcripts such as *sodB* (Massé *et al.*, 2002).

![mRNA Degradation](image)

**Fig 1.12: sodB mRNA degradation model for RyhB sRNAs**

Finally, another possible mechanism for sRNA action could involve the action of ribonuclease with the sRNA inhibiting its access by binding to and stabilising the mRNA (Storz *et al.*, 2004) (Fig 1.13).

![mRNA Stability](image)

**Fig 1.13: mRNA stability model with sRNAs**
Hfq could be a significantly important protein in the regulation of iron uptake. Washi et al. (1999) have demonstrated that an *E. coli* *hfq* cat mutant causes an increase in the level of expression of the outer membrane proteins FepA and FhuA, which are two of the proteins involved in the transport of iron in *E. coli*. As a result of this *hfq* mutation, iron accumulates in the cell leading to the appearance of hydroxyl radicals and to an increased sensitivity of the cell toward hydroxyl radicals. This suggests that under iron deplete conditions, Hfq is a negative regulator of the iron transport proteins FepA and FhuA.

The way Hfq regulates FepA and FhuA still remains to be understood. However, another outer membrane protein OmpA is also negatively regulated by Hfq. As explained before, the regulator binds to *ompA* mRNA and regulates its stability by competing with the ribosome and allowing the cleavage of the mRNA by RNase E. A similar mechanism could occur for the regulation of the stability of the two iron transport outer membrane proteins. It is likely that Hfq regulates these two outer membrane proteins at the post-transcriptional level, in fact, only these two outer membrane iron transport receptors are Hfq regulated while they all are Fur regulated, suggesting that Hfq functions independently of Fur, post transcriptionally.

The literature shows that a number of genes are up regulated by Fur. The first example of this unexpected regulation was the positive Fur control of iron regulated superoxide dismutase encoded by *sodB*. Superoxide dismutase functions to lessen the load of hydroxyl radicals in the cell, which are a source of oxidative damage. Fur mediated positive regulation was subsequently discovered for other proteins, such as the ferritins Bfr and Ftn, aconitase AcnA, and fumarase FumC. No Fur box was located in the promoter regions of these genes.

A recent study of the *sodB* promoter showed clearly that the mRNA is post-transcriptionally regulated (Dubrac et al., 2000). In a *fur* mutant, *sodB* mRNA half-
Life is about five min, while in the wild type it is fourteen min. Results of promoter-deletion analysis indicate that a palindrome and an AU-rich RNA region in the untranslated part of the sodB mRNA are important for Fur-dependent stabilization. The mystery of this regulation remained until sRNA was discovered.

Indeed, with the identification and characterisation of the sRNA RyhB, Masse et al. (2002) were able to demonstrate that under iron deplete conditions, Hfq together with RyhB, a Fur regulated small RNA, down regulate the level of some proteins, with some of them related to iron acquisition and metabolism. So far, six genes targeted by RyhB have been identified. Two of them clearly encode the iron-storage proteins, ferritin and bacterioferritin, thus releasing the iron bound to these proteins into the cytoplasm. These proteins have the purpose of preventing iron-dependent damage by removing free iron from the cytoplasm and are also used as a source of iron under iron deplete conditions. This stored iron can also help to repair damaged iron-containing proteins and repress oxidative damage. As well, three enzymes from the TCA cycle are down regulated by RyhB: succinate dehydrogenase encoded by the sdh operon, aconitase encoded by acnA, and fumarase encoded by fumA.

Masse et al. (2003) also established that RyhB causes the rapid degradation of its mRNA targets in a manner dependent on RNase E. In addition, RyhB itself is unstable under conditions of normal transcription when its transcripts are being made and rapidly degraded in an RNase E dependent manner. Masse's initial model was that stress signals cause induction of RyhB. Then, Hfq binds to the sRNA efficiently defending it from degradation by ribonuclease and presenting it to its targets. Moll et al. (2003) observed that RyhB sRNA has a half life >30 min in E. coli wild type while its stability is drastically dropped in a hfq mutant strain to fifteen minutes. Hfq also binds to the target mRNAs like sodB mRNA (Geissmann et al., 2004). It is not clear if Hfq leaves the sRNA-mRNA complex but either way, the complex is then rapidly degraded. It could be that the binding of Hfq to the RNA blocks access to RNaseE since the RNase E and Hfq recognition sites are matching. Hfq binding to RNA occurs particularly at AU-rich single stranded regions as does...
RNase E. The finding that RyhB is rapidly consumed during use provides a mechanism for the rapid recovery from iron starvation, and provides a clear demonstration of the use of a small RNA as a reversible regulatory switch.

Geissmann et al. (2004) have described the mechanism of interaction between RyhB and Hfq as shown in Fig 1.14.

**Fig 1.14: Model of sodB mRNA-Hfq-RyhB interaction.**

Hfq binds with high affinity to sodB mRNA, via an A/U-rich sequence preceding stem-loop b. This binding causes the mRNA to adopt a structure in which stem-loop b, which follows the Hfq-binding site, is opened out to give a large loop containing the translation start codon, which lies within the sequence complementary to RyhB. The stem of stem-loop b starts with the ribosome-binding site. In conditions of iron deficiency (Fur inactivated), RyhB is produced and is stabilised by binding to Hfq. RyhB interacts with sodB mRNA by base pairing in the region containing the complementary sequence. This base pairing both modifies the structure of the RNA molecule and blocks translation. Changes in the structure of stem-loop b may lead to the release of Hfq. The block of translation and the structural change render the RNA molecule susceptible to RNase cleavages. Numbering starts at the transcription start site. The translation start site of sodB is indicated by an arrow. Hfq-binding sites are shown in red, and sequences complementary between sodB and RyhB are shown in green. Regions affected by Hfq binding are shown in bold (Geissmann et al., 2004).
Finally, the fur mRNA itself was identified as a target for negative posttranscriptional regulation by Hfq (Vecerek et al., 2003). The synthesis of the transcriptional regulator Fur is inversely correlated with the synthesis of Hfq. This new level of iron acquisition control could explain how E. coli doubles its iron content during the transition from exponential to stationary phase (Abdul-Tehran et al., 1999). The Hfq-mediated inhibition of Fur synthesis and the reduced half-life in an hfq+ background could suggest a mechanism of Hfq action for fur mRNA identical to the one for ompA mRNA with Hfq binding to the mRNA in a way that the degradation with RNase E is facilitated.

### 14.3.4 Other examples of iron responsive post transcriptional regulation

Similar mechanisms exist at the posttranscriptional level of regulation between the eukaryotes and prokaryotes regarding the regulation of iron uptake, one of which involves aconitase. In eukaryotes, two isozymes of aconitase are available. In its [4Fe-4S] cluster form, cytosolic aconitase has the same activity as the mitochondrial enzyme but in its apoform, the protein called IRP (iron regulatory protein) binds specific mRNAs, either to stabilize the mRNA or to block its translation (Beinert et al., 1996). In fact, under iron deplete conditions, the enzyme loses its [4Fe-4S] cluster, thus losing its activity and so is now able to bind to mRNA (Cairo et al., 2002, Eisenstein et al., 2000).

In prokaryotes, the apoforms of aconitases from E. coli and B. subtilis were found to be involved in translational regulation (Alen et al., 1999, Tang et al., 1999). E. coli contains two major isozymes of aconitase, aconitase A and aconitase B (Jordan et al., 1999). Aconitase B is the major aconitase of the TCA cycle whereas aconitase A is a stress-induced enzyme (Varghese et al., 2003). The apoforms of both of the E. coli enzymes and the B. subtilis enzyme have been shown to specifically bind their related mRNAs, apparently in order to enhance translation (Alen et al., 1999, Tang et al., 1999). Some results indicate that E. coli aconitases may regulate sodA, which
encodes a superoxide dismutase, at the post-transcriptional level (Tang et al., 2002)
Also, strains lacking both aconitases are hypersensitive to redox-stress agents such as hydrogen peroxide raising the question of whether these enzymes may control expression of additional target genes Analysis of the activities of aconitase A and B under conditions of oxidative stress and iron depletion suggests that aconitase B is demetallated in a non-oxidative manner, indicating that its cluster occupancy is related to the iron status of the cell (Varguese et al., 2003) If this is the case, then the proportion of aconitase B able to bind RNA (apo-aconitase) may be directly related to the cellular iron status In addition, results suggest that posttranscriptional regulation by the level of iron also occurs in other bacteria such as Xanthomonas campestris (Wilson et al., 1998) and in P. aeruginosa (Somerville et al., 1999)

Another example of posttranscriptional regulation is in Vibrio aguillarum, in which RNAα was the first antisense RNA reported to be involved in iron regulation (Salinas et al., 1992) In V. anguillarum, regulation is governed by both negative and positive factors (Tolmasky et al., 1995) The negative regulators are Fur and RNA α (Tolmasky et al., 1994, Waldbeser et al., 1993, 1995)

RNAα is a 650 bp RNA encoded in the fatB coding region in the complementary strand and which is preferentially expressed under iron replete conditions RNAα transcription is Fur regulated, while iron plays a role in increasing the RNAα stability (Chen et al., 1996) In addition, the iron transport of the siderophore anguibactin is encoded by the fatA, fatB, fatC, and fatD genes FatA is the receptor for ferric anguibactin complexes (Actis et al., 1995), FatB is a membrane-located lipoprotein that shares domain homology with periplasmic binding proteins (Actis et al., 1995) and FatC and FatD are cytoplasmic integral membrane proteins (Koster et al., 1991) The presence of RNAα results in a reduction of FatA and FatB expression, probably by interaction between the polycistronic fatDCBA mRNA and RNAα (Waldbeser et al., 1993, Waldbeser et al., 1995) This change appears to enhance processing upstream of the fatA coding region, resulting in a concomitant inhibition of FatA synthesis and a degradation of the fatB region in this mRNA (Waldbeser et al., 1993, Waldbeser et al., 1995)
Finally, the last example of post transcriptional regulation in iron acquisition can be found in *Bradyrhizobium japonicum* in the Fur-like transcriptional regulator Irr which under iron replete conditions is inactivated due to binding to ferrochelatase (Hamza *et al.*, 2000, Qi *et al.*, 1999, Qi *et al.*, 2002). This will be discussed in more detail in the section 5.6.2.
1.5. Rhizobia iron uptake and the legume symbiosis

1.5.1 Introduction

Rhizobia belong to the alpha-proteobacteria group. Many of the genera in the group have little in common apart from their ability to induce N₂-fixing nodules. Examples are shown in Table 1. The rhizobia can interact with plants inducing nodules wherein the bacteria convert atmospheric nitrogen into ammonia. This then becomes a source of nitrogen for the plant.

The symbiosis between plants and rhizobia is largely limited to legumes. The species name of the microsymbionts indicates in most cases the corresponding host plant nodulated. Symbiosis is a species-specific process but the degree of host specificity is quite different among rhizobia (Young et al., 1989).

The acquisition of iron by these genera is very important, as iron is an important constituent of the nitrogenase complex, that catalyses nitrogen fixation. Also bacteroids (nitrogen endosymbionts) have an important requirement for iron due their respiratory mechanism employing abundant cytochromes and other electron donors, each with their own iron centers (Delgado et al., 1998)

Table 1.1: Example of species and biovars of rhizobia

<table>
<thead>
<tr>
<th>Rhizobial strain/species</th>
<th>Host legumes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sinorhizobium meliloti</em></td>
<td>Alfalfa</td>
</tr>
<tr>
<td><em>Rhizobium leguminosarum bv. viciae</em></td>
<td>Peas, lentils, vetches</td>
</tr>
<tr>
<td><em>Rhizobium leguminosarum bv. phaseoli</em></td>
<td>Beans</td>
</tr>
<tr>
<td><em>Bradyrhizobium japonicum</em></td>
<td>Soybeans</td>
</tr>
<tr>
<td><em>Rhizobium japonicum</em></td>
<td>Soybeans</td>
</tr>
</tbody>
</table>
15.2 Infection and nodulation of plants by rhizobia

The infection of legumes by rhizobia is a complex process diagrammatically represented in Fig 15. The symbiosis is initiated when flavonoids and other plant compounds induce the bacteria to produce a molecular signal, the Nod factor, which stimulates cell divisions in the root, resulting in nodule organogenesis.

When the *Rhizobium* has received the signal, it attaches itself to the root of the legume usually at young growing root hairs. Once on the root surface, the bacterium generates damage leading to root hair branching, deforming and curling. The young root hairs can be curled sufficiently to entrap bacterial cells in a pocket of host cell wall.

Initiation of infection then involves structural alterations of the root hair cell wall. The mechanism of hydrolysis of the cell wall remains unclear, it could either involve an enzymatic reaction of the bacterium or the use of plant mechanisms such as those used when epidermal cells grow out into root hairs.

Once the plant wall is hydrolysed, the *Rhizobium* enters the plant by invagination of the root cell wall to form an incipient tubule which extends by tip growth. This tubule, the infection thread, grows down the inside of the root hair and into the body of the root hair cell (epidermal cell). Rhizobia inside the infection thread replicate keeping the tubule filled with bacteria. If the infection thread exits the epidermal cell, it does so by fusing with the distal cell wall, resulting in the release of bacteria into the intercellular space between the epidermal cell and the underlying cell layer. Invagination and tip growth, similar to that seen at the beginning of infection thread growth, occurs at the underlying cell wall and a bacteria-filled thread propagates further towards the inner root cortex (Van Spronsen et al., 1994). The inward-growing infection thread network and the outward-growing nodule eventually meet. Branching of the thread occurs and it then enters the nodule primordium ensuring...
that a sufficient number of nodule cells are colonized. Bacteria eventually exit the
infection thread network, thereby entering the cytoplasm of nodule cells. They then
differentiate into bacteroids and fix atmospheric nitrogen (Gage et al., 2000, Oke et al., 1999).

In various rhizobial species, common and host specific nod genes have been
identified determining infection and nodulation of specific hosts (reviewed by Fisher
et al., 1992). With the exception of nodD, which is constitutively expressed, none of
the nod genes are expressed in free-living cultured cells. Expression is induced upon
exposure to plant exudates (Mulligan et al., 1985) and this induction is dependent on
NodD. Many of the inducing molecules that have been purified from plant exudates
have been identified as flavonoids, three ringed aromatic compounds. In alfalfa, the
most active inducers are flavones such as luteolin. The proteins NodA, NodB and
NodC are required for both root hair curling and cell division, while NodFE, NodH
and NodLMN, which are involved in host selection affect the location and tightness
of root hair curling and the efficiency and persistence of cell division (reviewed by
Long et al., 1989). The basic structure of Nod factors seems to be a β-1,4-linked
oligomer of N-acetylglucosamine with an N-acyl n-substitution on the non-reducing
end (Fisher et al., 1992). Individual rhizobial strains may make a family of factors
that vary slightly in length and/or substitution. Substitutions usually differ when
factors from different species are compared, which may account for host range
distinctions between species and biovars of rhizobia.

After the initiation of infection, bacteria must complete the penetration and
subsequent release into the host cells. This process requires the presence of specific
bacterial surface components and plant components that include amongst them
neutral glucans, lipopolysaccharides and charged heteropolysaccharide. Possible
roles for the extracellular polysaccharides include signaling, osmotic regulation,
recognition and defense, which function to present and/or disguise the bacterium
during invasion. Within plant cells, the bacteria differentiate to form bacteroids,
which are essentially subcellular organelles within which conditions are optimized for the expression, protection and function of the nitrogenase enzyme

In *Rhizobium*, the genes for nitrogen fixation are generally divided into two groups. The *nif* genes refer to those with homologues in free-living nitrogen fixing organisms such as *Klebsiella*, while *fix* genes refer to those required for symbiotic nitrogen fixation, but whose function is not known to be analogous to any free living system. The symbiotic activation of the *nif* genes is dependent on NifA (Szeto et al., 1987). Redox-dependent control of *nifA* expression occurs in response to *fixL* and *fixJ* which encode a two-component regulatory system that is oxygen responsive (Merrick et al., 1992).
Root Nodule Formation

Infection thread spreads into adjacent cells

Formation of an infection thread through which rhizobia enter root cells

Bacteroids are released from the infection thread

Root hair covered by free-living rhizobia

Root hair starts to curl

Fig 1.15: Invasion of the plant by rhizobia
(http://www.microbiologyonline.org.uk/forms/rhizobium.pdf)
1.6. The importance of iron in rhizobia

In the Rhizobium-legume associations, there is a massive demand for iron, the nodule being a veritable magnet for the metal. The single most abundant protein that the plant host makes in the nodule is leghaemoglobin, an iron protein. This is required to buffer oxygen and protect the oxygen labile nitrogenase complex. In the bacteria, nitrogenase and nitrogenase reductase contain FeS clusters and the former has the cofactor FeMoCo at the active site for N\textsubscript{2} reduction (Johnston et al., 2001).

1.6.1 The requirement for iron during nodule formation

The availability of iron in the soil depends upon pH and oxygen content. Its availability can affect the initiation of symbiosis. To start nodulation, the bacterium must first come into contact with the root of the appropriate leguminous host. Therefore, one limiting factor for the start of nodulation is the abundance of the bacterium in the rhizosphere. A bacterium, which can compete effectively for the limited iron available, will have a competitive advantage and consequently will predominate over those that are less competitive. Siderophore iron uptake may confer a selective advantage in soils with a low amount of bioavailable iron. The ability to use xenosiderophores (those produced by other organisms) is also an advantage. Rhizobia have usually developed specific siderophore iron uptake systems which function in the free-living state and which allow efficient colonisation of the rhizosphere.

Iron depletion was found to decrease nodule number and nodule mass in a number of legumes. Peanuts, which are grown under iron-deplete conditions in calcareous soils, fail to nodulate until given foliar iron application. Plants treated with exogenous iron produce a greater number of excisable nodules and carry greater nodule mass compared to untreated plants. The mechanism by which iron affects nodule number and mass is unknown, however, it was suggested that the iron deficiency exerts a
greater effect on the rhizobia which were consequently unable to acquire adequate amounts of iron from the plant (O’Hara et al., 1988)

16.2 The role of iron in nodule function

Bacteroids are enclosed in a membrane that is derived from the plant plasma membrane termed the peribacteroid membrane. More than one bacteroid may be enclosed by a single membrane generating a peribacteroid unit or symbiosome.

Nodules differ in morphology and vascularisation depending on the plant host and they can therefore be grouped into two distinct groups: determinate and indeterminate nodules.

A determinate nodule is ephemeral and lasts days or a few weeks. It has a short, predestined life-span. Consequently, new nodules are being formed as the root grows in the soil and others are being lost on older parts of the root system. Soybean nodules are of this type. The nodule is a spherical elaboration of the ground tissue system in the root cortex and has a specialised anatomy.

The second nodule type is illustrated by several legumes including alfalfa, clover and pea which form indeterminate nodules. These are called indeterminate in that meristematic activity is theoretically unlimited. This type of nodule is more elongated compared to the determinate type and is tumescent. In this case, the nodule has an apical meristem which functions for many months, continuously producing new cells, which become infected with bacteria from older cells. These nodules have a much more extensive vascular system which surrounds the nitrogen-fixing parenchyma that occupy the center of the nodule.
16.2.1 Nitrogenase

The enzyme nitrogenase catalyses the conversion of nitrogen gas to ammonia in nitrogen-fixing organisms. This enzyme consists of two metalloproteins and is highly conserved in sequence and structure among nitrogen-fixing bacteria. In legumes, it only occurs within the bacteroids. The reaction requires hydrogen as well as energy from ATP. The nitrogenase complex is sensitive to oxygen, becoming inactivated when exposed to it. This is not a problem with free-living, anaerobic nitrogen-fixing bacteria such as some *Clostridium* species. Free-living aerobic bacteria have a variety of different mechanisms for protecting the nitrogenase complex, including high rates of metabolism and physical barriers. *Azotobacter* overcomes the oxygen problem by having the highest rate of respiration of any organism, thus maintaining a low level of oxygen in its cells.

16.2.2 Leghaemoglobin and haem biosynthesis

In the *Rhizobium*-legume symbiosis, oxygen levels in the nodule are controlled with leghaemoglobin. This iron-containing protein has a similar function to that of haemoglobin, i.e., it binds to oxygen. It provides sufficient oxygen for the metabolic functions of the bacteroids but prevents the accumulation of free oxygen that would destroy the activity of nitrogenase. Leghaemoglobin seemed to be a truly symbiotic protein with the apoleghaemoglobin synthesised by the plant and the haem moiety synthesised by the bacterium.

However, a *B. japonicum* haem mutant defective in δ-aminolevulnic acid (ALA) synthase enzyme that is involved in the first step of bacterial haem synthesis was found to form fully effective nodules on soybeans (Guerinot et al., 1986). This result was in contrast to previous results for a *S. meliloti* hemA mutant, which was shown to form nodules that were incapable of nitrogen fixation on alfalfa (Leong et al., 1982).
O'Brien (1996) tried to find an explanation for these conflicting results. It is now known that the plant produces the haem for leghaemoglobin. On the other hand, rhizobia synthesise haem that is used for example in the cytochromes of the bacteroids. The difference in phenotypes is simply that, in *B. japonicum*, the host supplies the bacteria with the necessary ALA to allow them to grow, whereas with *S. meliloti*, ALA is either not made accessible to *S. meliloti* or it is not taken up (McGinnis et al., 1995). Therefore, *S. meliloti* is starved and cannot survive because of their failure to make any haem for its own respiration.

1 6 2 3 Ferritin

As said before, ferritin, an iron storage protein, is present in eukaryotes and prokaryotes. Ko et al. (1987) showed an inverse correlation between the age of the nodule and the amount of ferritin present. Phytoferritin has also been found to disappear with the appearance of leghaemoglobin.

1 6 3 Iron uptake in the nodule

The demand for iron is high in the nodule. However, the way bacteroids get their supply of iron while in the nodule is still not clear.

The role of siderophores in iron uptake in the nodule was studied using well-characterised strains and mutants of *S. meliloti* and *R. leguminosarum* bv *viciae*.

In each case, it was concluded that the siderophore was not contributing to the iron supply in the bacteroid (Lynch et al., 2001).

This implies that novel mechanisms exist to supply iron in the nodule. In 1996, Wittenberg et al. reported that most iron in the nodule was between the bacteroids and the peribacteroid membrane bound to molecules appearing to be of bacterial origin. It could be that this siderophore-like protein is only expressed in the
bacteroid (Fisher et al., 1994) Recent studies using macroarrays have identified nine new genes induced in mature nitrogen-fixing bacteroids (Ampe et al., 2003)

Alternatively, the bacteroid could acquire iron by taking up ferrous iron, which would not necessitate a siderophore. Indeed, the environment around the bacteroids, which in oxygen-deplete conditions due to leghaemoglobin would be expected to contain ferrous iron, and the peribacteroid membrane which possesses a ferri-chelate reductase could provide ferrous iron (LeVier et al., 1996)
Siderophore mediated iron transport in rhizobia

As discussed before, most iron is acquired by the bacteria through the use of siderophores. The development of the CAS assay by Schwyn et al. (1987) has greatly helped in the identification of bacterial siderophore production. One interesting discovery was that *B. japonicum* does not produce any siderophore that can be detected by the assay (Guerinot et al., 1990). In comparison, *Rhizobium* and *Sinorhizobium* species produce siderophores of which vicibactin and rhizobactin 1021 are the most extensively characterised.

1641 The *R. leguminosarum* siderophore vicibactin

*R. leguminosarum* biovar *viciae* produces a novel hydroxamate siderophore termed vicibactin (Dilworth et al., 1998). Vicibactin is transported in *R. leguminosarum* by a system similar to the Fhu system in *E. coli*. Eight genes, vbsGSO, vbsADL, vbsC, and vbsP were identified as genes involved in the biosynthesis of vicibactin (Carter et al., 2002). Upstream of those genes is *fhuA*, which encodes the outer membrane receptor used by this siderophore (Stevens et al., 1999).

1642 *S. meliloti* siderophores Rhizobactin and Rhizobactin 1021

Two different siderophores have been identified and characterized in *S. meliloti*. One is produced by *S. meliloti* 1021, which was named rhizobactin 1021 (Persmark et al., 1993) while rhizobactin is produced by *S. meliloti* DM4 (Smith et al., 1985).

Rhizobactin 1021 is chemically similar to aerobactin and schizokinen (Fig. 1.16). Schizokinen, a siderophore produced by *B. megaterium* is identical in its core structure to rhizobactin 1021, which differs only by the presence of an unusual fatty acid attachment, a (E)-2-decanoic acid residue (Persmark et al., 1993). Aerobactin is produced by *Aeromonas* sp. and various strains of pathogenic *E. coli* and *Shigella* sp. Aerobactin, which is structurally similar to rhizobactin 1021, is a known
virulence factor and has been found to be an important virulence determinant even in strains that produce other siderophores (Der Vartanian, 1988).

Fig 1.16: Chemical structure of siderophores

Reigh et al. (1993) identified a mutant defective in the synthesis and uptake of rhizobactin 1021. Later, the rhizobactin operon was characterised by Lynch et al. (2001). They identified eight genes involved in the regulation, biosynthesis, and transport of rhizobactin 1021. Six of these genes, named rhbABCDEF, function in the biosynthesis of the siderophore and were shown to constitute an operon that is repressed under iron-replete conditions. rhtA encodes the outer membrane receptor protein for the siderophore. Finally, rhrA encodes an AraC-like transcriptional regulator that up regulates genes involved in the biosynthesis and the transport of the siderophore under iron deplete conditions. The cluster of genes is located on the pSyma megaplasmid of S. meliloti 2011.
16.5 Iron Regulation in Rhizobia

16.5.1 Fur studies in rhizobia

The Fur protein has been studied in two members of the rhizobia. The *Bradyrhizobium japonicum* fur gene was identified based on functional complementation of an *E. coli* mutant (Hamza et al., 1999). This transcriptional regulator was also characterized in *R. leguminosarum* (Wexler et al., 2003).

The results presented by Wexler et al. (2003) and Hamza et al. (1999) for respectively *R. leguminosarum* and *B. japonicum* suggest that in contrast to the other genera the regulation of many iron-responsive genes in the rhizobia is not mediated by Fur. This indicates that Fur is not in those cases a general regulator but more a specific one for a few iron regulated genes.

16.5.2 The fur gene of *R. leguminosarum*

The *fur* gene of *R. leguminosarum* was identified by De luca et al. in 1998 as a single copy gene present on the chromosome. It was first suggested that a mutation of the gene was lethal to *R. leguminosarum* (De luca et al., 1998) as previously seen in other bacteria. However, Wexler et al. (2003) finally obtained a *fur* mutant by allelic exchange. The *R. leguminosarum* *fur* mutant was found through mobility shift assays, to be unaffected for the control of iron responsive genes. On the other hand, purified *R. leguminosarum* Fur was able to bind to a canonical ‘Fur box’ and could partially complement an *E. coli* fur mutant.

Also, in the *R. leguminosarum* genome, there are no ‘Fur boxes’ found 5' of putative promoter sequences in the expected regions.

However, recently, Diaz-Mireles et al. (2004) showed that mutation of the *fur* gene, in the presence of Mn²⁺, causes high-level expression of the *sitABCD* operon, which
is responsible of the transport of Mn$^{2+}$. Indeed, mobility shift assays showed that the purified *R. leguminosarum* Fur protein could bind to at least two regions near the *sitABCD* promoter region even if this DNA has no conventional consensus Fur-binding sequences (Fur boxes). These results suggest that Fur is in fact a Mur (manganese uptake regulator), which acts as a Mn$^{2+}$ responsive transcriptional regulator even if its gene product resembles Fur.

1653 *The fur gene of B. japonicum*

The *fur* gene was also identified in a single copy on the chromosome of *B. japonicum* by Hamza *et al.* (1999). In this organism, Fur controls the expression of *irr* which is a transcriptional regulator controlling the biosynthesis of haem (Hamza *et al.*, 2000). Also, *B. japonicum* Fur was able to complement an *E. coli* fur mutant and its homology to *E. coli* fur indicates that the cloned *B. japonicum* DNA encodes a structural and functional homologue of Fur. Therefore, this complementation also suggests that the *B. japonicum* Fur can down regulate genes in vivo. Further experiments were carried out to confirm this. *E. coli* extract containing overexpressed *B. japonicum* Fur were used to show that the protein can bind to a canonical ‘Fur box’ in the presence of Mn$^{2+}$, a metal mimicking ferrous iron (Hamza *et al.*, 1999, 2000, Friedman *et al.*, 2003).

In addition, Friedman *et al.* (2003) showed that *B. japonicum* Fur for the first time binds to a sequence disparate from the Fur box consensus. It binds a DNA sequence in the promoter of *irr* that differs from the Fur box and to which *E. coli* Fur cannot bind (Hamza *et al.*, 1998). *B. japonicum* Fur can maximally protect a 30-bp region in DNase I footprinting analysis including three imperfect direct repeat hexamers. Alignment of the Fur box consensus to the 30-bp protected region of the *irr* promoter does not give a better match than 7 of 19 residues and, this low match is predicted to occur with very high frequency (9 x 10$^5$ sites/strand for a genome of 9 x 10$^6$ bp), and so cannot be the basis of a binding site for Fur.
Friedman et al. (2003) showed that the affinity of \textit{B. japonicum} Fur for its target DNA increases in the presence of the metal. DNase I footprinting demonstrated that the binding by \textit{B. japonicum} Fur to its binding site within the \textit{irr} promoter is ferrous iron-dependent.

\textbf{1.6.6 RirA studies in the rhizobia}

In most cases, in bacteria, the biosynthesis genes of siderophores are iron responsive genes, the chelator being only produced under iron deplete conditions. \textit{R. leguminosarum} is no exception (Worsley et al., 2000). However, if siderophore expression is usually repressed in gram negative bacteria by the ferric uptake regulator Fur, this is not the case in \textit{R. leguminosarum}.

In \textit{R. leguminosarum}, a new transcriptional regulator called RirA (Rhizobial Iron Regulator) was identified (Todd et al., 2002). It seems to be a gene involved in iron regulation as a knock out mutation up-regulates a number of genes involved in iron metabolism.

Therefore, in \textit{R. leguminosarum}, it has been shown that Fur plays a less important role than in most other gram-negative organisms. Moreover, Todd \textit{et al}'s work (2002) have shown that in \textit{R. leguminosarum}, the \textit{rirA} mutation affects the expression of all promoters that are found to have an increased level of transcription under low iron conditions. Indeed, the gene mutation results in the high-level constitutive expression of at least eight operons whose transcription is normally iron-responsive and whose products are involved in the synthesis and uptake of vicibactin or in the uptake of haem and other iron sources. \textit{rirA} transcription is increased two-fold under iron replete conditions. Also, the \textit{R. leguminosarum} RirA N-terminal region shows significant matches with other transcriptional regulators suggesting that this is the DNA-binding domain of the protein (Todd \textit{et al}, 2002).
16.7 Specific transcriptional regulators in the rhizobia

16.7.1 Rpol in R. leguminosarum

In *R. leguminosarum*, studies revealed that the transcription of genes encoding the siderophore have an absolute requirement for a gene denoted *rpol*, located upstream from the vicibactin biosynthesis genes (Yeoman *et al.*, 1999). From sequence analysis, Rpol appears to be a member of the ECF (extra cytoplasmic factors) sigma factors of RNA polymerase but its mechanism of action still has to be determined. *rpol* itself is upregulated under iron-deplete conditions (Yeoman *et al.*, 1999) and by a mechanism involving RirA (Todd *et al.*, 2003).

The *R. leguminosarum* vbs operons involved in vicibactin biosynthesis are regulated by at least three different systems of gene control, distinguishable by their response to the availability of iron in the medium and the need for a functional Rpol factor. With the exception of *vbsP*, the vbs genes are transcribed at higher levels under iron-deplete conditions. Rpol is necessary for the expression of *vbsGSO* and *vbsADL* (Yeoman *et al.*, 1999) and binds the promoter regions of the *vbsGSO* and *vbsADL* operons (Yeoman *et al.*, 2003). In contrast, expression of *vbsC* is iron regulated, but the adjacent *rpol* is not involved in its transcription.

16.7.2 Irr in B. japonicum

Regulation of iron homeostasis in bacteria has focused on Fur. However, Irr (iron response regulator) from the bacterium *B. japonicum* mediates iron control of haem biosynthesis. Irr was identified in 1998 (Hamza *et al.*, 1998). Irr from *B. japonicum* is a Fur-like protein but still quite different from Fur. For instance, its gene expression is iron regulated while *fur* is essentially constitutive. Iron represses the *irr* gene moderately at the transcriptional level and strongly at the level of protein turnover (Hamza *et al.*, 1998, Qi *et al.*, 1999). The latter mechanism involves iron-
dependent binding of haem to a haem regulatory motif of the Irr protein, which is necessary for its degradation (Qi et al., 1999) As a result, irr mRNA is reduced but is detectable under high iron conditions, while protein levels are undetectable Hamza et al. (2000) showed that irr is Fur regulated, mobility shift assays showing that Fur binds to its promoter region However, irr can respond to iron in a fur mutant strain B. japonicum must have a mechanism for sensing and responding to the cellular iron level in addition to Fur Haem mediates iron-dependent degradation of Irr (Qi et al., 1999) and so haem may be the form of iron to which Irr responds

Hamza (1998) isolated an irr mutant, which under iron deplete conditions accumulated protoporphyrin, a precursor of haem biosynthesis and which showed high expression of hemB encoding a haem synthesis enzyme The hemA gene is also controlled by iron (Page et al., 1994), but is regulated by Fur (Hamza et al., 2000) B. japonicum is the only organism described so far containing a Fur-like protein in addition to a traditional Fur involved in iron metabolism (Hamza et al., 1999)

1673 Additional uncharacterised iron regulators in Rhizobia

Analysis of R. leguminosarum and B. japonicum suggest the presence of further unidentified iron regulators

A laboratory strain of R. leguminosarum was found to have acquired a mutation that affected iron responsive gene regulation (De luca et al., 1998) Although the phenotype associated with this strain was similar to the one described for a rirA mutant, the mutation was not located in this gene (Todd et al., 2002)

A palindromic repeat sequence was identified between the hmuR and hmuT genes of B. japonicum that function in haem utilisation Mutagenesis of the repeat sequence led to a drastic reduction in hmuT and hmuR gene expression The reduction in expression was shown to be unrelated to the activity of the Irr or Fur protein, suggesting regulation by an as yet unidentified regulator (Nienaber et al., 2001)

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1.7. Summary

This thesis is dedicated to the study of iron regulation in *S. meliloti* 2011 using the siderophore mediated iron uptake system as the main focus.

In most gram-negative bacteria, the general iron regulation is mediated through the ferric uptake regulator Fur, however, a new kind of iron regulator was identified in another member of rhizobia *R. leguminosarum* and denoted the rhizobial iron regulator RirA (Todd *et al.*, 2002). The homologues of these two proteins were identified and characterised in *S. meliloti* and the results of these studies are described respectively in chapter 3 and 4.

In addition, specific regulation of iron uptake can occur. Previous work (Lynch *et al.*, 2001) has shown that the rhizobactin 1021 uptake system is also regulated by the AraC-like transcriptional regulator RhrA. Furthermore, post transcriptional regulation of the outer membrane receptor encoded by *rhtA* apparently takes place (O’Connell, personal communication). An analysis of the regulation by RhrA and of its binding was performed and is described in chapter 4.

Finally, *S. meliloti* is an agriculturally important soil bacteria forming a nitrogen-fixing symbiosis with alfalfa, which is known to be subject to luteolin regulation via the NodD genes. Recently, it has been shown that flavonoids can affect the expression of genes which are not the *nod* genes and in the absence of ‘Nod box’ in their promoter regions (Perret *et al.*, 1999, Chen *et al.* 2000). Thus, the luteolin regulation of the genes involved in the siderophore regulon was assessed and this work is described in chapter 5.
Chapter 2:
Materials and Methods
2.1 Bacterial strains and plasmids

The bacterial strains, primer sequences and plasmids used in this study are described in tables 2.1, 2.2 and 2.3 respectively.

Table 2.1: Bacterial strains

<table>
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<th>Strain</th>
<th>Phenotype/Genotype</th>
<th>Source/Reference</th>
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<tr>
<td><strong>Sinorhizobium meliloti</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>Wild type, Nod+ Fix+</td>
<td>Meade et al., 1982</td>
</tr>
<tr>
<td>2011rhhA62</td>
<td>Tn5lac insertion in rhhA</td>
<td>Lynch et al., 2001</td>
</tr>
<tr>
<td>2011rhhG25</td>
<td>G212 lacZ mutant with Tn5lacZ insertion in rhhG</td>
<td>Lynch et al., 2001</td>
</tr>
<tr>
<td>2011rhrA26</td>
<td>Tn5lac insertion in rhrA</td>
<td>Lynch et al., 2001</td>
</tr>
<tr>
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<td>Tn5lac insertion in rhtA</td>
<td>Lynch et al., 2001</td>
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<tr>
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<td>Lynch et al., 2001</td>
</tr>
<tr>
<td>2011rirA2</td>
<td>Kanamycin insertion in rirA</td>
<td>This study</td>
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<tr>
<td><strong>Escherichia coli</strong></td>
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<tr>
<td>DH5α</td>
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<td>Bethesda Research</td>
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<tr>
<td>JM109</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 (rK-mK^+), supE44, relA1 .(lac^- proAB^+)[F' traD36 proAB lacFΔM15].</td>
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<td>XL1-blue</td>
<td>recA1, hsdR17 (rK12-mK12^+), supE44, lac, [F' .proA^+B^+ lacF, lacZΔM15 Tn10 (Tet^R)]</td>
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<tr>
<td>Strain</td>
<td>Phenotype/Genotype</td>
<td>Source/Reference</td>
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<td>XL10-gold</td>
<td>Tet $^R$, $\Delta$(mcrA)183, $\Delta$(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-I, recA1, gyrA96, relA1, lac Hte[F’ proAB lacF’Z ΔM15 Tn10 (Tet$^R$ ) Amy Cam$^R$ ]</td>
<td>Stratagene</td>
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<tr>
<td>Rosetta blue</td>
<td>EndA, hsdR17($r_{K12}$=$m_{K12}$+), supE44, thi-I, recA1, gyrA96, relA, lac [F’ proA'B'lacF'ZΔM15::Tn10(Tet$^R$)] pRARE</td>
<td>Novagen</td>
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<tr>
<td>H1681</td>
<td>Thr, se,r fhuA, lacy, rpsL, galK, hsdR, mcrA, fhuF::LAMpLacMU fur-31 zbf::Tn10</td>
<td>Braun et al. (1990)</td>
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<td>INVaF'</td>
<td>F', recA1, hsdR17($r_{K12}$=$m_{K12}$+) supE44, φ80lac ZΔM15, Δ(lacZYA-argF)U169</td>
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Table 2.2: Primers

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<tr>
<th>Primer name</th>
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<tr>
<td><em>rirA</em>-F</td>
<td>CTCGAG TCG CCG AGG CCC ATT CCT TCT</td>
</tr>
<tr>
<td><em>rirA</em>-R</td>
<td>ACTAGT GAA GTC GGC TGT AAA CGG TAT GCG</td>
</tr>
<tr>
<td><em>KanNcoI</em>-F</td>
<td>CCATGG GAC GTT GTA AAA CGA CGG CCA GTG</td>
</tr>
<tr>
<td><em>KanNcoI</em>-R</td>
<td>CCATGG GGA AAC AGC TAT GAC CAT GAT TAC G</td>
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<td><em>Fur</em>-F</td>
<td>ACC ATT CCC CCG GTT ACG CTG ATC</td>
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<tr>
<td><em>Fur</em>-R</td>
<td>CGT CGG CCT CGC TCA AGG AGT C</td>
</tr>
<tr>
<td><em>KanBss</em>-F</td>
<td>GCGCGC GAC GTT GTA AAA CGA CGG CCA GTG</td>
</tr>
<tr>
<td><em>KanBss</em>-R</td>
<td>GCGCGC GGA AAC AGC TAT GAC CAT GAT TAC G</td>
</tr>
</tbody>
</table>

*Promoter probes*

| F-*rhrAWT* | CCC. AAGCTT CCC TGG AGG CGT CCT ATC GCC |
| R-*rhrAWT* | AAAA CTGCAG GGC AAC ATT GTC TGA CGA TAA ACA TG |
| F-*rhrAM1* | TTT AAGCTT TAC TGT CTT AAT GAG GTT CGC TCA C |
| F-*NcoIPOT1* | CAGT CCATGG GCA AAT GGG ATT GCC |
| F-*EcoRIpOT1* | CG GAAATTC ATT ATT TGT AGA GCT CAT CC |
| R-*BglIIIR2* | GA AGATCT TCA CAT CCA AGC CGT TC |
| F-*BglIIIR2* | GA AGATCT TCA CAT CCA AGC CGT TC |
| F-*BglIII+6* | GA AGATCT GTT CGC TCA CAT CCA AGC CGT TC |

*Mobility Shift Assay*

| MSARHTX-F | CGGGATCC CCT ATC GCC TCT CTC GAA AAT GC |
| MSARHTX-R | CGGGATCC CGA AAA CTG CCA CTG CCC GGC |
| MSAheme-F | CGGGATCC GGA CCA GTC CTT TGA AAG TGT TGG |
| MSAheme-R | CGGGATCC GTT TTC TTA TGT GAC GAA AAT AAG GC |
| MSAsitA-F | CGGGATCC CCC GCG ACA CTA GCC AAG GGG |
| MSAsitA-R | CGGGATCC CCG GCT CTC CTC TTT GCG AAC C |
| MSArhra-F | CGGGATCC GTC GTG CGC CAG CCT TTC CTG |
| MSArhra-R | CGGGATCC T GCC CAT AA CGC CCC CTG CGC |

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<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSAfhuF-F</td>
<td>CGGGATCC CGG AAC GAT AGG CCA TAA TCG GG</td>
</tr>
<tr>
<td>MSAfhuF-R</td>
<td>CGGGATCC TCC CCA GCC ACT GCC CAG CG</td>
</tr>
</tbody>
</table>

**Protein Cloning**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>RhrA60-F</td>
<td>CCATGG AGACAATCCGACCG</td>
</tr>
<tr>
<td>RhrA60-R</td>
<td>GGATCCAGCGGAGCTGCCAG</td>
</tr>
<tr>
<td>Fur60-F</td>
<td>CCATGG AGAGCCAGAGCAAGAATCGGATCG</td>
</tr>
<tr>
<td>Fur60-R</td>
<td>GGATCC GTC CTGCGCTTCGCAATAG</td>
</tr>
</tbody>
</table>

**Real-time RT-PCR**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>RhbA-F</td>
<td>ATG CCG GCC GAT TTA GCC</td>
</tr>
<tr>
<td>RhbA-R:</td>
<td>TCG CGT CTT TCC TGT CGG</td>
</tr>
<tr>
<td>RhtA-F</td>
<td>CTATGGAATTTGGCAACTACTC</td>
</tr>
<tr>
<td>RhtA-R</td>
<td>CGATGATCTCAACGGCAAGC</td>
</tr>
<tr>
<td>RhrA-F</td>
<td>TGC CAG CGA CAG GGA AAC G</td>
</tr>
<tr>
<td>RhrA-R:</td>
<td>ATG GAG ACA ATC CGA CCG</td>
</tr>
<tr>
<td>dppA1-F:</td>
<td>CAC TAC TCT CTT GGC AGC G</td>
</tr>
<tr>
<td>dppA1-R</td>
<td>ACG GCT GTA AAC GGT ATG CG</td>
</tr>
<tr>
<td>rirA-F:</td>
<td>GCG TCT GAC GAA GCA AAC C</td>
</tr>
<tr>
<td>rirA-R</td>
<td>GCG TCT GAC GAA GCA AAC</td>
</tr>
<tr>
<td>16S rRNA-F:</td>
<td>ACT TGA GAG TTT GAT CCT GGC</td>
</tr>
<tr>
<td>16S rRNA-F:</td>
<td>TCT TTC CCC CGA AGG GCT C</td>
</tr>
<tr>
<td>npt-F:</td>
<td>CGC AGG TTC TCCGCGC GCG</td>
</tr>
<tr>
<td>npt-R:</td>
<td>CTG CGC AAG GAA CGC CCG</td>
</tr>
<tr>
<td>Smc02726-F:</td>
<td>ATGCTCAACCGGCATCGCCTGGCC</td>
</tr>
<tr>
<td>Smc02726-R:</td>
<td>CGCGAGATCTCTCTTCAGCAGGCTCG</td>
</tr>
</tbody>
</table>
### Table 2.3: Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pOT1</td>
<td>Wide-host-range <em>gfp</em> promoter-probe plasmid, <em>Gm</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Allaway <em>et al.</em>, (2001)</td>
</tr>
<tr>
<td>pMP220</td>
<td>Wide-host-range <em>lacZ</em> promoter-probe plasmid; <em>Km</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Spaink <em>et al.</em>, (1987)</td>
</tr>
<tr>
<td>pCR2.1</td>
<td>PCR Cloning Vector: <em>Amp</em>&lt;sup&gt;R&lt;/sup&gt;, <em>Km</em>&lt;sup&gt;R&lt;/sup&gt;, <em>lacZ</em>&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pUC4K</td>
<td><em>Amp</em>&lt;sup&gt;R&lt;/sup&gt;, Source of <em>Km</em>&lt;sup&gt;R&lt;/sup&gt; cassette</td>
<td>Amersham</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pBR322</td>
<td><em>Tec</em>&lt;sup&gt;R&lt;/sup&gt;, Source of <em>Tec</em>&lt;sup&gt;R&lt;/sup&gt;cassette</td>
<td>Roche</td>
</tr>
<tr>
<td>pJQ200sk+</td>
<td><em>Gm</em>&lt;sup&gt;R&lt;/sup&gt;, <em>sacB</em>, mob</td>
<td>Quandt <em>et al.</em>, (1993)</td>
</tr>
<tr>
<td>pRK600</td>
<td><em>Cm</em>&lt;sup&gt;R&lt;/sup&gt;, pRK2013 Nm::Tn9, provides transfer functions</td>
<td>Finan <em>et al.</em>, 1986</td>
</tr>
<tr>
<td>pSTBlue-1</td>
<td>Cloning Vector: <em>Amp</em>&lt;sup&gt;R&lt;/sup&gt;, <em>Km</em>&lt;sup&gt;R&lt;/sup&gt;, <em>lacZ</em>&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Novagen</td>
</tr>
<tr>
<td>pQE60</td>
<td>High copy number expression vector</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pRARE</td>
<td><em>Cm</em>&lt;sup&gt;R&lt;/sup&gt; (<em>ArgU, arg W, ile X, glyT, leuW, proL</em>) to improve overexpression yield</td>
<td>Novagen</td>
</tr>
</tbody>
</table>

**pCR2.1 Derived vectors**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTAFur</td>
<td>500 bp <em>NcoI/BamHI</em> product encoding Smc02510 for overexpression</td>
<td>This study</td>
</tr>
<tr>
<td>pTARhrA</td>
<td>1 Kb bp <em>NcoI/BamHI</em> product encoding RhrA for overexpression</td>
<td>This study</td>
</tr>
<tr>
<td>pTAKanNcoI</td>
<td><em>Km</em>&lt;sup&gt;R&lt;/sup&gt; cassette as an <em>NcoI</em> fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pTAFurM</td>
<td>2.2 Kb bp <em>BamHI/NotI</em> fragment encoding for Smc02510 for mutagenesis</td>
<td>This study</td>
</tr>
<tr>
<td>pTARirAM</td>
<td>2.2 Kb bp <em>XhoI/SpeI</em> fragment encoding for RirA for mutagenesis</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Source/Reference</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>PSTblue-1 Derived vectors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSTfur</td>
<td>2.2 Kb bp <em>BamHI/NotI</em> fragment encoding for Smc02510 for mutagenesis</td>
<td>This study</td>
</tr>
<tr>
<td>pSTfurTec</td>
<td>Tec gene inserted into the <em>BssHII</em> site of <em>smc02510</em> in pSTfur</td>
<td>This study</td>
</tr>
<tr>
<td>pJQ200ks+ Derived vectors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pJQrirA</td>
<td>2.2 Kb bp <em>XhoI/Spel</em> fragment in pJQ200ks+ encoding for RirA for mutagenesis</td>
<td>This study</td>
</tr>
<tr>
<td>pJQrirAK</td>
<td>Kanamycin cassette in the <em>NcoI</em> site of <em>rira</em> in pJQrirA</td>
<td>This study</td>
</tr>
<tr>
<td>pJQnrfA</td>
<td>2.2 Kb bp <em>Spel/NotI</em> fragment in pJQ200ks+ encoding for NrfA for mutagenesis</td>
<td>This study</td>
</tr>
<tr>
<td>pJQnrfAK</td>
<td>Kanamycin cassette in the <em>BssHII</em> site of <em>rira</em> in pJQnrfA</td>
<td>This study</td>
</tr>
<tr>
<td>pJFurTc</td>
<td>3.6 Kb bp <em>Spel/NotI</em> fragment in pJQ200ks+ encoding for Smc02510 with a tetracycline cassette into the <em>BssHII</em> site of the gene for mutagenesis</td>
<td>This study</td>
</tr>
<tr>
<td>pOT1 Derived vectors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWT</td>
<td><em>HindIII/PstI</em> promoter region of <em>rhtX</em> in pOT1</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Source/Reference</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>pM1</td>
<td>HindIII/PstI promoter region of rhtX in pOT1 without first repeat</td>
<td>This study</td>
</tr>
<tr>
<td>pEN2</td>
<td>HindIII/PstI promoter region of rhtX in pOT1 without sec repeat</td>
<td>This study</td>
</tr>
<tr>
<td>pEN3</td>
<td>HindIII/PstI promoter region of rhtX in pOT1 without both repeats</td>
<td>This study</td>
</tr>
<tr>
<td>pEN4</td>
<td>HindIII/PstI promoter region of rhtX in pOT1 with extended intergenic region between the repeats</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td><em>pQE60 Derived vectors</em></td>
<td></td>
</tr>
<tr>
<td>pRhrA60</td>
<td>Ncol/BamHI fragment encoding RhrA cloned into pQE60 for overexpression</td>
<td>This study</td>
</tr>
<tr>
<td>pFur60</td>
<td>Ncol/BamHI fragment encoding Smc02510 cloned into pQE60 for overexpression</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.2 Microbiological Media

Solid complex media contained 15 g/L Oxoid No 3 agar. Tryptone and yeast extract were from Oxoid. Other chemicals were from Sigma Chemicals Co Ltd and BDH Chemicals Ltd. All chemicals were analar grade. All minimal and low iron media were prepared in ultra pure water. Distilled water was used to prepare complex media and sterilisation was achieved by autoclaving at 15 lb/ in\(^2\) for 20 min.

♦ TY Medium (Beringer, 1974)

Used for the routine culturing of *S. meliloti* strains

- Tryptone: 5 g
- Yeast extract: 3 g
- CaCl\(_2\) 2H\(_2\)O: 0.7 g

Adjusted to pH 7.0 with NaOH and volume brought to 1 l with dH\(_2\)O. The solution was then sterilised by autoclaving.

♦ Luria Bertani Broth (LB) (Sambrook et al., 1989)

Used for the routine culturing of *E. coli* strains

- Tryptone: 10 g
- Yeast extract: 5 g
- NaCl: 10 g

Adjusted to pH 7.0 with NaOH and volume brought to 1 l with dH\(_2\)O. The solution was then sterilised by autoclaving.
After making LB and autoclaving as described above, MgSO₄ and KCl were added to final concentrations of 4 mM and 10 mM respectively.

**SOB Medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
<tr>
<td>KCl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>dH₂O</td>
<td>1 l</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

After autoclaving, the solution was allowed to cool to 55°C and sterile solutions of MgCl₂ (1M) and Mg₂SO₄ (1M) were added to final concentrations of 10 mM.

**SOC Medium**

After making SOB as above, 7.2 ml of 50% sterile glucose was added to give a final concentration of 20 mM.

**Jensen Plant Media (Jensen, 1942)**

Used for nodulation analysis of *Medicago sativa*

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar No 3</td>
<td>7.5 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>550 ml</td>
</tr>
</tbody>
</table>

Following autoclaving solutions of K₂HPO₄, Mg SO₄ and NaCl were added to a final concentration of 0.2% also added was CaHPO₄ to a final concentration of 0.1% and
FeCl₃ to a final concentration of 0.01%. Each of these solutions was autoclaved separately.

- **MacConkey Medium**

Used for the β-galactosidase assay.

Mac Conkey agar N°3 51.5 g

The powder was dissolved in 1 l dH₂O and the solution was then sterilised by autoclaving.

- **Low iron Media**

All low iron media were prepared with ultra pure water and supplemented with the appropriate concentration of 2,2'-dipyridyl.

### 2.3 Solutions and Buffers

- **TE Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>10 mM</td>
</tr>
<tr>
<td>Na₂-EDTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
</tr>
</tbody>
</table>

- **TES Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>10 mM</td>
</tr>
<tr>
<td>Na₂-EDTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>50 mM</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
</tr>
</tbody>
</table>
♦ STET Buffer (Holmes and Ouigley, 1981)

Tris-HCl 50 mM (5 ml of a 1M solution)
Na₂-EDTA 50 mM (10 ml of 0.5 M solution)
Triton X-100 5% (v/v)
Sucrose 8% (w/v)
dH₂O to 100 ml
pH 8.0

♦ Solutions for the 1,2,3 Plasmid DNA preparation method (Birnboim and Doly, 1979)

**Solution 1**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1 ml (0.5 M solution)</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>0.25 ml (of a 1M solution)</td>
</tr>
<tr>
<td>Na₂-EDTA</td>
<td>1 ml (of 0.1 M solution)</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 10 ml</td>
</tr>
</tbody>
</table>

**Solution 2**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>2 ml (of 1 M solution)</td>
</tr>
<tr>
<td>SDS</td>
<td>1 ml (of 10% solution)</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 10 ml</td>
</tr>
</tbody>
</table>

Made up every month and stored at room temperature

**Solution 3**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium acetate</td>
<td>3 M</td>
</tr>
<tr>
<td>pH</td>
<td>4.8</td>
</tr>
</tbody>
</table>
To 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of dH2O was added. The resulting solution was 3 M with respect to potassium and 5 M with respect to acetate.

♦ 50X Tris acetate (TAE) Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>100 ml (of 0.5 M solution)</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>57.1 ml</td>
</tr>
<tr>
<td>Tris</td>
<td>242 g</td>
</tr>
<tr>
<td>dH2O</td>
<td>to 1 l</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Diluted to 1X with dH2O before use.

♦ 6X Gel Loading dye

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromophenol Blue</td>
<td>0.25 %</td>
</tr>
<tr>
<td>Xylene Cyanol</td>
<td>0.25 %</td>
</tr>
<tr>
<td>Ficoll (Type 400)</td>
<td>15 %</td>
</tr>
</tbody>
</table>

Made in dH2O and stored at room temperature following autoclaving.

♦ Solutions for Competent Cells

*TB Buffer for competent cells (Inoue et al., 1990)*

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipes</td>
<td>10 mM</td>
</tr>
<tr>
<td>CaCl2</td>
<td>15 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>250 mM</td>
</tr>
<tr>
<td>pH with KOH</td>
<td>6.7</td>
</tr>
</tbody>
</table>

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Once the pH had been adjusted, MnCl$_2$ was added to a final concentration of 55 mM. The solution was then filter sterilised through a 0.45 μm sterile filter and stored at 4°C.

**TFB1 Buffer for competent cells**

- RbCl: 100 mM
- MnCl$_2$: 50 mM
- Potassium acetate: 30 mM
- CaCl$_2$: 10 mM
- Glycerol: 15%
- pH: 5.8

The solution was filter sterilised through a 0.45 μm sterile filter and stored at 4°C.

**TFB2 Buffer for competent cells**

- MOPS: 10 mM
- RbCl: 10 mM
- CaCl$_2$: 75 mM
- Glycerol: 15%
- pH with KOH: 6.8

The solution was filter sterilised through a 0.45 μm sterile filter and stored at 4°C.
• Solutions for Southern Blot Analysis

20XSSC

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>175.83 g</td>
</tr>
<tr>
<td>Trisodium citrate</td>
<td>88.2 g</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 1 l</td>
</tr>
</tbody>
</table>

Denaturing solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>87.66 g</td>
</tr>
<tr>
<td>NaOH</td>
<td>20 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 1 l</td>
</tr>
</tbody>
</table>

Neutralising solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>87.66 g</td>
</tr>
<tr>
<td>Tris</td>
<td>121.1 g</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 1 l</td>
</tr>
</tbody>
</table>

Washing Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maleic Acid</td>
<td>11.61 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.76 g</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.3% (v/v)</td>
</tr>
<tr>
<td>pH</td>
<td>7.5 (with solid NaOH)</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 1 l</td>
</tr>
</tbody>
</table>
Maleic Acid Buffer

Maleic Acid 11.61 g
NaCl 8.76 g
pH 7.5 (with solid NaOH)
dH2O to 1 l

Detection Buffer

Tris 12.11 g
NaCl 5.84 g
pH 9.5
dH2O to 1 l

Denhardt's solution (50X)

Ficoll (Type 400) 5 g
Polyvinylpyrrolidone 5 g
BSA (Pentax Fraction V) 5 g
dH2O 500 ml

Salmon Sperm DNA

Salmon sperm DNA was dissolved in water at a concentration of 10 mg/ml, and mixed until dissolved. The DNA was sheared by passing it several times through an 18-gauge hypodermic needle. The DNA was boiled for 10 min, dispensed into small aliquots and stored at -20°C.
**Prehybridisation solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSC</td>
<td>6X</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5% (w/v)</td>
</tr>
<tr>
<td>Denhart's solution</td>
<td>5X</td>
</tr>
<tr>
<td>Salmon Sperm (10 mg/ml)</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Salmon sperm DNA was prepared as the prehybridisation solution by boiling for 5 min and chilling quickly in an ice water bath.

**Hybridisation solution**

Hybridisation solution was prepared as the prehybridisation solution above and denatured labelled probe was added.

**10 X Block stock solution**

Blocking Reagent 10% (w/v)

The blocking reagent was dissolved under constant stirring in Maleic acid buffer and heated to 65°C. The solution remained opaque. To prepare 1 X blocking solution, the blocking stock was diluted with Maleic acid buffer.

◆ Antibody Solution

The antibody was centrifuged at 10,000 rpm for 5 min before each use. The antibody was diluted 1:5000 (150 mU/ml) in blocking solution.
Solution for SDS/gel electrophoresis

Separating gel

<table>
<thead>
<tr>
<th></th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>4.1 ml</td>
<td>3.4 ml</td>
<td>2.4 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Acrylamide/Bis-acrylamide (30% / 0.8% w/v)</td>
<td>3.3 ml</td>
<td>4.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005 ml</td>
<td>0.005 ml</td>
<td>0.005 ml</td>
</tr>
<tr>
<td>Total</td>
<td>10.005 ml</td>
<td>10.005 ml</td>
<td>10.005 ml</td>
</tr>
</tbody>
</table>

Stacking gel

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>3.075 ml</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>0.025 ml</td>
</tr>
<tr>
<td>Acrylamide/Bis-acrylamide (30% / 0.8% w/v)</td>
<td>0.67 ml</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate</td>
<td>0.025 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005 ml</td>
</tr>
<tr>
<td>Total</td>
<td>5.05 ml</td>
</tr>
</tbody>
</table>

The ammonium persulfate must be prepared on the day...
**5 X Running Buffer**

Tris Base  
Glycine  
SDS  
\[pH\ 8.3\]  

Add dH\(_2\)O to 1 l

**Sample Buffer**

\[\begin{array}{l}
dH\_2O & 4.0\ ml \\
0.5\ M \ Tris-HCl & 1.0\ ml \\
Glycerol & 0.8\ ml \\
10\%\ SDS & 1.6\ ml \\
\beta\text{-mercaptoethanol} & 0.4\ ml \\
0.05\% \ (w/v) \ Bromophenol\ blue & 0.2\ ml \\
\end{array}\]

The samples have to be diluted at least 1:4 and heated at 95°C for 5 min prior to loading

**Staining solution**

\[\begin{array}{l}
Acetic\ acid & 100\ ml \\
dH\_2O & 450\ ml \\
Methanol & 450\ ml \\
Bromophenol\ blue & 2.5\ g \\
\end{array}\]
**Destaining solution**

- Acetic acid 10 ml
- dH$_2$O 450 ml
- Methanol 450 ml

◊ Solutions for protein overexpression and purification

**Lysis buffer for overexpression of RhrA**

- 150 mM potassium/Acetate
- 10 mM Tris-acetate (pH 7.4)
- 1 mM EDTA

**Buffers for purification under denaturing conditions**

**Lysis buffer (1 l)**

- 100 mM NaH$_2$PO$_4$ 13.8 g
- 10 mM Tris Cl 1.2 g
- 8 M urea 480.5 g

Adjust pH to 8.0 using NaOH

**Wash buffer (1 l)**

- 100 mM NaH$_2$PO$_4$ 13.8 g
- 10 mM Tris Cl 1.2 g
- 8 M urea 480.5 g

Adjust pH to 6.3 using HCl
### Elution buffers (1 l)

<table>
<thead>
<tr>
<th>Buffer Component</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM NaH$_2$PO$_4$</td>
<td>13.8 g</td>
<td></td>
</tr>
<tr>
<td>10 mM Tris Cl</td>
<td>1.2 g</td>
<td></td>
</tr>
<tr>
<td>8 M urea</td>
<td>480.5 g</td>
<td></td>
</tr>
</tbody>
</table>

Adjust pH to 4.5 using HCl.

Due to the dissociation of urea, the buffers should be adjusted immediately prior to use. Do not autoclave.

### Buffers for purification under native conditions

#### Lysis buffer (1 l)

<table>
<thead>
<tr>
<th>Buffer Component</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM NaH$_2$PO$_4$</td>
<td>6.90 g</td>
<td></td>
</tr>
<tr>
<td>300 mM NaCl</td>
<td>17.54 g</td>
<td></td>
</tr>
<tr>
<td>10 mM imidazole</td>
<td>0.68 g</td>
<td></td>
</tr>
</tbody>
</table>

Adjust pH to 8.0 using NaOH.

#### Wash buffer (1 l)

<table>
<thead>
<tr>
<th>Buffer Component</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM NaH$_2$PO$_4$</td>
<td>6.90 g</td>
<td></td>
</tr>
<tr>
<td>300 mM NaCl</td>
<td>17.54 g</td>
<td></td>
</tr>
<tr>
<td>150 mM imidazole</td>
<td>1.36 g</td>
<td></td>
</tr>
</tbody>
</table>

Adjust pH to 8.0 using NaOH.

86
*Elution buffer (1 l)*

50 mM NaH₂PO₄  6.90 g  
300 mM NaCl  17.54 g  
250 mM imidazole  17.00 g

Adjust pH to 8.0 using NaOH

♦ Solutions for Electrophoretic mobility shift assay (EMSA)

*PolydIdC stock*

PolydIdC was aliquoted in 1 mg/ml stocks in polydIdC dilution buffer (10 ml TE with 200 μl 5M NaCl)

*5 X Binding Buffer for Fur EMSA (Ochsner et al, 1995)*

- bis-tris Borate (pH 7.5)  50 mM
- KCl  200 mM
- MgSO₄  1 mM
- Glycerol  10%

Add before use

- PolydIdC  50 μg/ml
- BSA  0.1 mg/ml
- MnSO₄  0.1 mM
**10 X Running Buffer for Fur EMSA (Ochsner et al., 1995)**

bis-tris Borate (pH 7.5) 200 mM

Due to rapid oxidation, MnSO₄ was added on the day to a concentration of 0.1 mM

**4 X Binding Buffer for RhrA EMSA (Hendrickson et al., 1984)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-acetate (pH 7.4)</td>
<td>40 mM</td>
</tr>
<tr>
<td>100 mM KCl</td>
<td>200 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>4 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>20%</td>
</tr>
</tbody>
</table>

Add before use:
- BSA 50 µg/ml
- DTT 1 mM
- Poly(dIdC) 50 µg/ml

**10X TBE Running Buffer for RhrA EMSA**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>108 g</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>55 g</td>
</tr>
<tr>
<td>0.5 M EDTA (pH 8.0)</td>
<td>40 ml</td>
</tr>
</tbody>
</table>

* Solution for Miller assay

**LacZ buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄</td>
<td>16.1 g</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>5.5 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.75g</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.246g</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>2.7 ml</td>
</tr>
<tr>
<td>DH₂O</td>
<td>1 L</td>
</tr>
</tbody>
</table>
Solution for native electrophoresis gel for EMSA

The formula used to calculate the volume of Accugel [40% (29 1) Acrylamide Bisacrylamide solution] used to prepare a gel of a given percentage is as follows

- \( V_a = \) volume of accugel to be used (ml)
- \( V_t = \) Total volume of gel casting solution required (ml)
- \( X = \% \) gel desired

\[ V_a = (V_t)(x) \]

\[ 40 \]

<table>
<thead>
<tr>
<th>Solution</th>
<th>4%</th>
<th>5%</th>
<th>8%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accugel</td>
<td>5 0 ml</td>
<td>6 2 ml</td>
<td>10 0 ml</td>
</tr>
<tr>
<td>10x TBE for RhrA EMSA / 10x Fur EMSA Running buffer</td>
<td>5 0 ml</td>
<td>5 0 ml</td>
<td>5 0 ml</td>
</tr>
<tr>
<td>dH₂O (Ultrapure)</td>
<td>39 9 ml</td>
<td>38 7 ml</td>
<td>34 9 ml</td>
</tr>
<tr>
<td>Total</td>
<td>50 ml</td>
<td>50 ml</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

Then 5 \( \mu \)l of 1 M DTT was added to the gel mixture followed by 50 mg of ammonium persulfate and 15 \( \mu \)l of TEMED. The gel mixture swirled briefly and poured into the gel mould. The comb was then inserted and the gel allowed to set for at least 45 min.

2.4 Antibiotics

Antibiotics used were from Sigma Aldrich Co Ltd. Antibiotics were prepared to a concentration of 100 mg/ml and stored in the dark at -20°C unless otherwise indicated.
Ampicillin was prepared in dH2O and used at a final concentration of 100 μg/ml in solid and liquid broth for *E. coli*

Chloramphenicol was prepared in ethanol and used at a final concentration of 20 μg/ml in both solid and liquid media.

Tetracycline was prepared in 50% ethanol at a concentration of 10 mg/ml. Tetracycline was used at a final concentration of 10 μg/ml for *S. meliloti* and *E. coli* in both liquid and solid media.

Kanamycin was prepared in dH2O. For *S. meliloti*, kanamycin was used at a final concentration of 100 μg/ml in solid media and 50 μg/ml in liquid broth. For *E. coli*, kanamycin was used at a final concentration of 30 μg/ml in both solid and liquid media.

Gentamicin was prepared in dH2O. For *S. meliloti* and *E. coli*, gentamicin was used at a final concentration of 20 μg/ml in both solid and liquid media.

Streptomycin was prepared in dH2O and used at a final concentration of 1 mg/ml in solid media for *S. meliloti*.

### 2.5 Storing and culturing bacteria

Strains were stored as glycerol stocks. A 1 ml aliquot of a late log phase culture was added to 0.5 ml of sterile 80% glycerol in a microfuge, which was then mixed and stored at -20°C. A duplicate set of long term stocks were stored at -80°C. Where hosts are harbouring plasmids, the appropriate antibiotic was added to the stock medium. Working stocks were stored on plate at 4°C.
2.6 Plasmid preparation method by the 1,2,3 Method.

This method was described by Birnboim and Doly (1979) A 1.5 ml aliquot of a bacterial culture grown in selective media was pelleted at 13,000 rpm in a microfuge and the supernatant was removed. The pellet was resuspended by vortexing in 200 μl of solution 1 and was then left for 5 min at room temperature. Then 200 μl of solution 2 was added, the tube was mixed by inversion and placed on ice for 5 min. Then 200 μl of solution 3 was added, the tube was mixed by inversion and placed on ice for 10 min. A clot of chromosomal DNA formed and was pelleted by centrifugation at 13,000 rpm in a microfuge for 10 min. The supernatant was then placed in a fresh tube and 600 μl of phenol chloroform isoamylalcohol (25 24 1) was added and mixed by vortexing. After centrifugation at 13,000 rpm for 5 min the aqueous layer was removed to a fresh tube and equal volume of isopropanol was added. After mixing, the tube was incubated at room temperature for 10 min. Then the tube was centrifuged at 13,000 rpm for 10 min to pellet the plasmid DNA. The pellet was washed with 70% ethanol, dried briefly in a vacuum dryer and resuspended in 50 μl of TE buffer. Plasmid DNA was stored at 4°C.

2.7 Plasmid Preparation By the Rapid Boiling Method.

This method was described by Holmes and Quigley (1981) and used instead of the 1,2,3 procedure outlined above for the screening of large numbers of transformants. A 1.5 ml aliquot of an overnight culture was spun at 13,000 rpm in a microfuge for 5 min and the supernatant removed. The pellet was resuspended in 350 μl of STET buffer. A 20 μl aliquot of 10 mg/ml lysozyme solution (prepared fresh in STET buffer) was added and the microfuge tube incubated at 30°C for 10 min. The tube was then placed in a boiling water bath for 60 sec and then spun at 13,000 rpm, for 10 min. The supernatant was removed to a fresh tube and an equal volume of isopropanol was added. The tube was left at room temperature for 10 min and then the plasmid DNA was pelleted by centrifugation at 13,000 rpm for 10 min.
pellet was washed with 70% ethanol, dried briefly in a vacuum dryer and then dissolved in 50 μl of TE buffer. Plasmid DNA was stored at 4°C.

2.8 Preparation of total genomic DNA from *S. meliloti*

A 1.5 ml aliquot of early stationary phase culture of *S. meliloti* was pelleted at 13,000 rpm for 5 min. The cells were washed with 1.5 ml of TES buffer and resuspended in 700 μl of TE buffer. Lysozyme solution (20 mg/ml in TE) was prepared freshly and 50 μl was added and the suspension was incubated at 30°C for 20 min. A sarkosyl/pronase solution (10% sarkosyl in TE containing 5 mg/ml pronase) was prepared and 50 μl was added and the suspension incubated at 37°C for one hr. Lysis was evident by an increase in the viscosity of the suspension. Sodium acetate (70 μl of a 3 M solution) was added and mixed gently. Then 600 μl of phenol, chloroform, isoamylalcohol (25:24:1) was added and the suspension was mixed slowly by inversion for 5 min. After centrifugation at 13,000 rpm for 5 min, the aqueous phase was removed to a fresh centrifuge tube and 600 μl of phenol, chloroform, isoamylalcohol (25:24:1) was added again and mixed slowly by inversion for 5 min. Following centrifugation at 13,000 rpm for 5 min, the supernatant was removed to a fresh centrifuge tube. Phenol extraction was carried out by adding 700 μl of chloroform, isoamylalcohol (24:1), mixing by inversion for 5 min, and by centrifugation at 13,000 rpm for 5 min. The aqueous layer was removed to a fresh microfuge tube and the DNA was precipitated with an equal volume of isopropanol and was evident in the suspension as a coiled thread. The microfuge tube was spun at 13,000 rpm for 10 min to pellet the DNA. The pellet was washed twice with 70% ethanol, air-dried and dissolved in 200 μl of TE buffer. Genomic DNA was stored at 4°C.
2.9 Agarose gel electrophoresis for DNA characterisation

DNA was analysed by agarose gel electrophoresis. Gels were prepared by dissolving agarose in 1 X TAE buffer to the required concentration (typically 0.7-2.0 %) and boiling until the solution became translucent. The 1 X TAE buffer was also used as the running buffer. A tracker dye was incorporated into DNA samples to facilitate loading of samples. Mini-gels were frequently run at 140 Volts for 20-30 min or until the tracker dye had migrated the required distance while maxi gels were frequently run at 40 Volts overnight. Gels were stained by immersing in a bath of ethidium bromide for 20 min and then destained by immersing in a water bath for 10 min. Gels were then visualised on a UV transilluminator and photographed using a UV image analyser.

2.10 Phenol/Chloroform extraction and ethanol precipitation

Phenol/chloroform extraction and ethanol precipitation was carried out to concentrate nucleic acid samples or change the buffers in which a sample was dissolved. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the DNA solution, mixed by vortexing and centrifuged for 5 min at 13,000 rpm. The upper aqueous phase was removed, taking care not to take any material from the interphase, it was placed in a sterile microfuge tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the aqueous phase, vortexed as before and centrifuged for 5 min at 13,000 rpm. Again the upper aqueous phase was removed to a fresh tube. One-tenth volume of 3 M sodium acetate (pH 5.2) was added to the solution of DNA, mixed and then 2 volumes of 100 % (v/v) ethanol were added. This mixture was vortexed and incubated at room temperatures for 5 min. The DNA samples were then centrifuged for 30 min at 12,000 rpm at 4°C, the supernatant was removed and pellets were washed with 1 ml 70 % (v/v) ethanol to remove excess salts. The tube was centrifuged for 5 min at 10,000 rpm, the
supernatant was removed and pellets were air dried for approximately 10 min. Pellets were resuspended in an appropriate volume of sterile Tris-EDTA (TE) (pH 8.0) or dH₂O.

2.11 Restriction digestion of DNA

The restriction enzymes used were supplied with incubation buffers at a concentration of 10X (working concentration 1X). DNA was digested with restriction endonucleases for identification purposes or to linearise or cut fragments from a plasmid. DNA digests were performed by adding:

- 200 ng – 1 μg of DNA (Final concentration of <300 ng/μl)
- 1 μl of enzyme/μg of DNA solution (10 U)
- 10 X buffer to a final concentration of 1X
- dH₂O to the final volume required

The reaction was gently mixed, centrifuged, and then incubated for 2 hrs at the optimum enzyme temperature (between 37°C and 50°C, usually 37°C).

2.12 PCR and TA Cloning of PCR Products.

<table>
<thead>
<tr>
<th>Standard PCR reaction Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
</tr>
<tr>
<td>Primers (0.6nm/μl)</td>
</tr>
<tr>
<td>Buffer (10X)</td>
</tr>
<tr>
<td>dNTP Mix (10 mM)</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
</tr>
<tr>
<td>RedTaq DNA polymerase</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>
Standard PCR Program

Stage 1:
Step 1: 95°C for 10 min

Stage 2:
Step 1: 95°C for 1 min
Step 2: Annealing temperature for 30 sec
Step 3: 72°C for 1 min for every Kb to be synthesised.
(Stage 2 was repeated for 30 cycles)

Stage 3:
Step 1: 95°C for 10 min

PCR products were routinely cloned using Original TA cloning Kit vector pCR2.1 from Invitrogen. The diagram below shows the concept behind the TA cloning method (Fig 2.1).

![Diagram of TA cloning](image)

Fig 2.1: Principle of TA cloning

The method is dependent on the fact that thermostable polymerases like Taq DNA polymerase lack 3'-5' exonuclease activity, leave 3' A-overhangs. PCR products generated with Taq DNA polymerase have a high efficiency of cloning in the TA cloning system. Other thermostable polymerases like Vent and Pfu, which have 3'-5' exonuclease activity, do not leave 3' A-overhangs.
PCR products were amplified using a standard PCR reaction mixture and using RedTaq DNA polymerase from Sigma. They were subsequently ligated with the TA pCR2.1 vector. The ligation was set as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh PCR product</td>
<td>1 µl</td>
</tr>
<tr>
<td>PCR2.1 Vector (25 ng/µl)</td>
<td>2 µl</td>
</tr>
<tr>
<td>10 X Ligation Buffer</td>
<td>1 µl</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>5 µl</td>
</tr>
<tr>
<td>T4 DNA ligase (4.0 Weiss U)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

The reaction was then incubated at room temperature overnight. Following incubation, 2-5 µl of the ligation was used to transform either *E. coli* DH5α cells prepared by the high efficiency method or *E. coli* INVαF' one shot competent cells that were supplied with the TA cloning kit.

To transform INVαF' cells, the cells were first thawed on ice. Then 2 µl of β-mercaptoethanol (0.5 M) was added and mixed gently with the pipette tip. Between 2-5µl of the ligation reaction mixture was added to the cells and mixed gently with the pipette tip. The cells were incubated on ice for 20 min and then 250 µl of SOC medium was added. The cells were incubated at 37°C for 1 hr. A 50 µl aliquot of the transformation mixture was plated on LB agar containing ampicillin (100 µg/ml) and X-gal to select the transformants and to test for α-complementation of the β-galactosidase. In addition to an ampicillin resistance gene the TA pCR2.1 vector also carries a kanamycin resistance gene. Kanamycin (30 µg/ml) was thus added to select for transformants instead of ampicillin when PCR products amplified from ampicillin resistant plasmids were being cloned.
2.13 Additional enzymatic reactions

RNase

RNase that was free of DNase was dissolved at a concentration of 10 mg/ml in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl. The solution was then dispensed into aliquots and stored at -20°C.

Klenow reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>18 μl</td>
</tr>
<tr>
<td>DNTPs (0.5 mM)</td>
<td>1 μl of each</td>
</tr>
<tr>
<td>Klenow Buffer (10X)</td>
<td>3 μl</td>
</tr>
<tr>
<td>Sterile dH2O</td>
<td>4 μl</td>
</tr>
<tr>
<td>Klenow (0.5U/μl)</td>
<td>1 μl</td>
</tr>
</tbody>
</table>

The reaction was incubated at room temperature for 1 hr. The reaction mixture was then phenol extracted to remove the enzyme and the DNA was ethanol precipitated.

Klenow labelling reaction

Probes were prepared as follows. Restricted DNA was boiled for 5 min and then chilled on ice water. A labelling reaction was then set up as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>15 μl</td>
</tr>
<tr>
<td>DNTPs labelling mix</td>
<td>2 μl</td>
</tr>
<tr>
<td>Hexanucleotide mix</td>
<td>2 μl</td>
</tr>
<tr>
<td>Klenow enzyme</td>
<td>1 μl</td>
</tr>
</tbody>
</table>
As longer incubation times resulted in an increase in labelling efficiency, the mixture was generally incubated for up to 20 hours. The probe was denatured by boiling for 10 min and chilling quickly on wet ice.

2.14 Preparation of ethidium bromide

A 10 mg/ml stock solution of ethidium bromide was prepared by dissolution in dH2O. The solution was stored in the dark at 4°C. A 100 µl aliquot of this stock solution was added to 1 l of dH2O for staining agarose gels. Gloves were worn at all times when handling solutions containing ethidium bromide. Ethidium bromide waste was collected and filtered through a deactivating filter (Schleicher and Schuell). The clear liquid was disposed of normally and the solids contained in the filter were incinerated.

2.15 Isolation of DNA from agarose gels

DNA was purified from agarose gels using a DNA gel purification kit (Eppendorf). The kit was used according to the manufacturer's instructions. Briefly, the gel slice was excised with a sterile scalpel and weighed. Three volumes of gel solubilizing buffer were added and the tube was incubated at 55°C until the gel slice had completely dissolved. One volume of isopropanol was added to the tube and mixed vigorously. Then, 800 µl of the solution was transferred into a spin cup and spun at 13,000 rpm for 1 min. The flow through was discarded and 750 µl of washing solution was added and spun for a further min at 13,000 rpm. The flow through was again discarded and the cup was again spun at 13,000 rpm for 2 min. The spin cup was transferred to a fresh microfuge tube and 30 µl of TE was added. The cup was then spun at 13,000 rpm for 1 min to elute the DNA.

2.16 Preparation of high efficiency competent cells.

This method was described by Inoue et al. (1990). A frozen stock of the appropriate E. coli strain was thawed, streaked on LB agar and incubated at 37°C overnight. Approx. 10-12 large colonies were removed with an inoculating loop and inoculated.
in 250 ml of SOB medium in a 2 l baffled flask. The culture was grown at 18°C with vigorous shaking (200-250 rpm) until OD$_{600}$ of 0.6 was reached. The flask was then placed on ice for 10 min. The culture was transferred to a 250 ml centrifuge bottle and spun in a Beckmann J2-21 centrifuge at 5,000 rpm and 4°C for 5 min. The pellet was resuspended in 80 ml of ice-cold TB buffer, placed on ice for 10 min and spun down as before. The cell pellet was gently resuspended in 20 ml of ice-cold TB buffer and DMSO was added slowly with gentle swirling to a final concentration of 7%. After incubation in an ice bath for 10 min the cell suspension was dispensed in 1 ml aliquots into microfuge tubes. The cells were then flash frozen in liquid nitrogen and stored at -80°C. Cells prepared in this manner frequently gave transformation efficiencies of the order of 10$^8$-10$^9$ transformants/μg DNA which is comparable with those attainable by electroporation.

2.17 Transformation of high efficiency competent cells.

A microfuge tube of cells prepared according to the procedure outlined in section 2.16 was allowed to thaw on ice and a 1-5 μl aliquot of plasmid preparation was added to 200 μl of the competent cells. The contents of the tube were briefly mixed and incubated on ice for 30 min. The cells were heat shocked at 42°C for 30 sec and then transferred back onto ice for 2 min. Then 0.8 ml of SOC medium was added and the cells were incubated at 37°C with vigorous shaking for 1 hr. A 100 μl aliquot of the resulting transformation mixture was plated on appropriate selective media and the plates were incubated at 37°C overnight.

2.18 Preparation of competent cells by RbCl treatment.

A frozen stock of the appropriate E. coli strain was thawed, streaked on LB agar and incubated at 37°C overnight. A single colony was picked and a 10 ml LB broth was inoculated and incubated at 37°C overnight. One ml of the overnight culture was added to 100 ml of LB broth and grown shaking at 37°C until and OD$_{600}$ of 0.5 was
reached. The flask was then placed on ice for 5 min. The culture was transferred to a centrifugation bottle and spun in a Beckman J2-21 centrifuge at 5,000 rpm and 4°C for 5 min. The cell pellet was carefully resuspended in 30 ml of ice cold TFB buffer, incubated on ice for 90 min and spun down as before. The cell pellet was gently resuspended in 4 ml of ice cold TFB2 and the cell suspension was dispensed in 1 ml aliquots into sterile microfuge tubes. The cells were then flash frozen in liquid nitrogen and stored at -80°C.

2.19 Transformation of competent cells prepared by RbCl treatment

A microfuge tube of cells prepared according to the procedure outlined in section 2.18 was allowed to thaw on ice and a 10 μl aliquot of the ligation or plasmid was added to 100 μl of the competent cells. The contents of the tube were briefly mixed and incubated on ice for 20 min. The cells were heat shocked at 42°C for 90 sec and then transferred back onto ice for 2 min. Then 0.5 ml of Pst broth medium was added and the cells were incubated at 37°C with vigorous shaking for 60 to 90 min. A 100-200 μl aliquot of the resulting transformation mixture was plated on an appropriate selective medium and the plates were incubated at 37°C overnight.

2.20 Bacterial conjugation by triparental mating

*S. meliloti* was grown to late log phase in TY, while *E. coli* donors were grown to mid log phase in LB broth. *E. coli* donors (0.75 ml) were mixed with an *E. coli* (0.75 ml) strain carrying the mobilising plasmid pRK600. The mixture was then pelleted at 13,000 rpm for 3 min, resuspended in 100 μl of fresh LB and then spotted onto the centre of an LB plate. Following incubation overnight at 37°C, the bacteria were resuspended in 3 ml of LB broth. Then 0.75 ml of the mated bacterial donor and helper cultures was mixed with 0.75 ml of the *S. meliloti* recipient culture and the mixture was pelleted as above. The pellet was resuspended in 100 μl of TY broth and spotted onto the centre of a TY plate. Following incubation overnight, the bacteria were resuspended in 2 ml of TY broth and dilutions were plated on appropriate media.
selective media. As controls, the donor mix and the recipient strain were spotted separately on agar plates and carried through the procedure as outlined above. Donor and recipient strains were then plated on the appropriate selective media.

### 2.21 Southern blot analysis

Following electrophoresis, the gel was stained in a bath of ethidium bromide and photographed. The DNA was denatured by immersing the gel in a denaturing solution and agitating gently at room temperature for 1 hr. The gel was subsequently immersed in a neutralising solution, and incubated with gentle agitation at room temperature for 1 hr. A gel tray was inverted in a bath of 20 X SSC, and a sheet of Whatman 3 MM paper cut to the width of the gel was soaked in the 20 X SSC and placed on top of the gel tray, with the ends dipping into the solution forming a wick. Air bubbles were removed by gently rolling the Whatman paper with a glass rod. The gel was inverted and placed gently on top of the Whatman paper. A piece of nitrocellulose filter cut exactly to the size of the gel was placed onto the surface of 2 X SSC and allowed to soak from beneath. The filter was immersed in the solution for a further 2 min, and then placed on top of the gel. Air bubbles were removed as described above. Three pieces of Whatman paper were cut to the size of the gel and two of them were soaked in 2 X SSC and placed on top of the filter. The third piece was then placed on top. Air bubbles were removed as described above. A stack of paper towels approx. 20 cm high was placed on top of the Whatman paper, ensuring that the towels did not come in contact with the wicks, and a weight was placed on top. The transfer of DNA was allowed to proceed for approximately 12-24 hrs. (See Fig 2.2)
Following the completion of the transfer, the paper towels and the Whatman paper on top of the gel were removed and the gel and the filter were placed gel side up on a dry sheet of Whatman paper. The positions of the wells were marked on the filter, which was then soaked for 5 min in 6 X SSC. The filter was allowed to dry at room temperature for 1 hr and it was subsequently placed between two sheets of Whatman paper and baked at 80°C for 2 hrs to irreversibly bind the DNA to the filter. The filter was then wrapped in Whatman paper and stored until required.

The filter was placed in roller bottles and at least 20 ml of prehybridisation solution was added per 100 cm² of filter. The filter was incubated while rotating for 1 hr. The prehybridisation solution was removed and hybridisation solution was added. The filter was incubated rotating for at least 16 hrs. Following hybridisation, the filter was washed twice at room temperature with 2 X SSC / 0.1 % SDS for 5 min. The filters were subsequently washed twice at 65°C with periodic agitation with 0.1 X SSC / 0.1 % SDS for 30 min.
Immunological detection was performed using the DIG DNA labelling and Detection Kit from Roche. Briefly, a 100 cm² filter was washed in washing buffer for 5 min. The filter was then incubated in 100 ml of blocking solution, which was prepared freshly for at least 1 hr. Then, the filter was incubated for 20 min with 20 ml of antibody solution. The filter was washed twice for 15 min with washing buffer and then equilibrated for 5 min in 20 ml of detection buffer. The filter was incubated with 10 ml of colour substrate solution and incubated in the dark until colour development was complete. The colour reaction was stopped by washing with TE buffer.

2.22 Surface sterilisation of *Medicago sativa*

*Medicago sativa* seeds were washed with sterile water and then stood in ethanol for 5 min. The ethanol was poured off and the seeds were again washed in sterile water. The water was poured off and the seeds were again washed with sterile water. The water was poured off and the seeds were washed in domestic bleach for 10 min. The bleach was then poured off and the seeds were washed four times with sterile water. The seeds were then spread on TY plates and incubated at room temperature in the dark for two days.

2.23 Nodulation Analysis of *Medicago sativa*

Two day old seedlings were transferred to Jensen medium and inoculated with approx. $10^5$ *S. meliloti* by streaking on the surface of the media. The plants were incubated for 30 days, after which they were observed for nodulation and assayed for nitrogen fixation.

2.24 Analysis of nitrogen fixation by gas chromatography

Nitrogen fixation was assayed by the acetylene reduction assay (Wacek and Brill, 1976). Nodules were excised and placed into a sterile suba sealed vessel. The
atmosphere was then made 10% with respect to acetylene. Acetylene reduction was determined by gas chromatography using a Poropak N column and a flame ionisation detector following a 24 hrs incubation period. The injector temperature was 70°C and then the oven temperature was 120°C.

2.25 Protein overexpression

Recombinant protein overexpression was carried out with *E. coli* strains harbouring the *lacP* mutation to produce enough lac repressor to efficiently block transcription. The *E. coli* expression cultures were grown in LB broth. Overnight cultures were used to inoculate 100 ml LB broth supplemented with the appropriate antibiotics. The culture was grown until the OD$_{600}$ reached 0.3-0.6. The culture was then induced with IPTG to a suitable final concentration. The culture was incubated for the appropriate time depending on the protein overexpressed. A sample or the whole culture was harvested by centrifugation at 6,000 rpm for 5 min. The pellets were kept for as long as a month at −20°C. A culture grown in the same conditions but without induction was used as a negative control.

2.26 Purification.

The pellet was resuspended in sonication buffer and sonicated on ice for the adequate time. The lysate was centrifuged at 10,000 rpm for 20 min (at 4°C for native preps) to pellet the cellular debris. The correct amount of resin was added to the clear lysate. The final mixture was shaken on a belly dancer at 4°C for native preps and at room temperature for denatured preps for 1 hr. The mixture was then centrifuged at 10,000 rpm for 1 min. The flow through was saved for SDS-PAGE analysis of the purification. The resin was then washed twice with the adequate volume of washing buffer, each wash was saved and every time the pellet was centrifuged for 1 min. Finally, the protein was eluted 3 times with elution buffer, the eluates were collected to be analysed by SDS-PAGE and then pooled together before further treatment.
2.27 Preparation of dialysis tubing

The dialysis tubes were rinsed in distilled water and placed in a beaker filled with distilled water. One spatula of EDTA was added to it. The water was heated until ebullition and then boiled for a further 2 min. The liquid was allowed to cool down and then removed. The tubes were then rinsed with more distilled water, the tubes were stored in water at 4°C until utilisation.

2.28 Protein SDS-PAGE electrophoresis

A protein gel electrophoresis system was used in this study. Glass plates were washed with detergent, rinsed first with tap water and then with ddH$_2$O and finally wiped in one direction with tissue soaked with 70% ethanol. The gasket was placed about the ridged plate, the plates were put together and secured with clamps. The depth of the resolving gel was marked on the outer plate. The resolving gel was then poured to within 2 cm of the top of the larger plate and overlaid with isopropanol. When set, the isopropanol was removed and the stacking gel was poured. A clean comb was inserted and the gel was allowed to polymerise for 45 min-1 hr. The electrophoresis tank was filled with 1 X running buffer to the level of the horizontal rubber gasket. After polymerisation the gaskets, clamp, stands and comb were removed. Unpolymerised gel was removed by gently rinsing the wells with ddH$_2$O, the wells were then straightened using a loading tip. The prepoured gels were lowered into the buffer at an angle to exclude air bubbles from the gel buffer interface. The gel plates were fixed firmly in place with the notched plate innermost. The chamber formed by the inner plates was filled with 1X running Buffer, the samples were loaded and the electrodes were attached. The gels were electrophoresed at a constant current of 25 mA per gel. When complete the plates were removed, separated and the gel was stained in Coomassie blue. Staining took place for 30 min, agitating constantly. The gel was then placed in destain in destaining buffer with constant agitation, until all background staining was removed. The destaining buffer was changed as it became saturated with stain.
2.29 Electrophoretic Mobility Shift Assay (EMSA) or Bandshift Assay

**Preparation of the probes**

The probes were made by PCR using genomics preps from *E. coli* H1681 and *S. meliloti* 2011 as templates. The PCR products were cut by the *BamH*I enzyme (generating 5' protruding ends suitable for the subsequent labelling reaction with T4 polynucleotide kinase). The cut PCR products were then dephosphorylated. Removal of 5' phosphate groups was carried out by treatment of DNA with Calf Intestinal Phosphatase (CIP). DNAs (< 100 ng/µl) were dephosphorylated using CIP in a 100 µl volume (CIP was added at 1 U/100 pmoles for cohesive termini). The solution was mixed gently and incubated for 30 min at 37°C. This was followed by an enzyme denaturation step achieved by heating to 75°C for 10 min. DNA was then purified by phenol/chloroform extraction and ethanol precipitation.

The labelling reaction was performed by adding:

- 30 pmol of substrate DNA containing 5'-hydroxyl termini
- 10 X kinase buffer to give a final concentration of 1 X
- 50 pmol of γ-32P dATP (4000 Ci/mmol, 10 mCi/ml)
- 20 U T4 polynucleotide kinase
- dH2O to a final reaction volume of 50 µl

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (30 pmoles)</td>
<td>X µl</td>
</tr>
<tr>
<td>Buffer (10 X)</td>
<td>1.66 µl</td>
</tr>
<tr>
<td>γ-32P dATP</td>
<td>5.00 µl</td>
</tr>
<tr>
<td>T4 polynucleotide kinase</td>
<td>1.33 µl</td>
</tr>
<tr>
<td>H2O</td>
<td>X µl</td>
</tr>
<tr>
<td>Total</td>
<td>16.66 µl</td>
</tr>
</tbody>
</table>
The reaction was incubated at 37°C for 40 min. Then 0.3 μl of 0.5 M EDTA were added and the mixture mixed, after which end-labeled oligonucleotides were purified away from incorporated labeled nucleotides by spin-column chromatography through MicroSpin™ G-25 columns essentially according to manufacturer's specifications (Amersham Pharmacia Biotech) or by ethanol precipitation.

**Binding reaction**

*Fur EMISA binding reaction*

The binding of purified proteins or extracts to labeled DNA probe was performed in a reaction comprising:

**Binding Mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding reaction buffer (5 X)</td>
<td>4 μl</td>
</tr>
<tr>
<td>Non-specific competitor DNA poly dI-dC (1 μg/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>BSA (1 mg/ml)</td>
<td>2 μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>7 μl</td>
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**Binding reaction**

<table>
<thead>
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<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding Mix</td>
<td>7 μl</td>
</tr>
<tr>
<td>³²P-labeled DNA probe (20,000 cpm/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Purified protein/Extract</td>
<td>X μl</td>
</tr>
<tr>
<td>Tris/Cl pH 8</td>
<td>X μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20 μl</td>
</tr>
</tbody>
</table>
A control reaction lacking purified proteins/extracts but containing all other components of the binding reaction was also set up. The reaction components were mixed gently and incubated at 30°C at room temperature for 20-25 min. The samples were then loaded onto a 5% polyacrylamide gel. One extra lane with Bromophenol blue was also added so that the leading edge of the gel was visualised. The gels were first prerun for 20 min at 200 Volts and then for 2-2.5 hrs at 200 Volts.

**RhrA EMSA binding reaction**

The binding of purified proteins or extracts to labeled DNA probe was performed in a reaction comprising:

<table>
<thead>
<tr>
<th>Binding Mix</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding reaction buffer (4 X)</td>
<td>5 μl</td>
</tr>
<tr>
<td>Non-specific competitor DNA poly dl-dC (1 μg/μl)</td>
<td>2 μl</td>
</tr>
<tr>
<td>BSA (1 mg/ml)</td>
<td>2 μl</td>
</tr>
<tr>
<td>Total</td>
<td>9 μl</td>
</tr>
</tbody>
</table>

**Binding reaction**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding Mix</td>
<td>9 μl</td>
</tr>
<tr>
<td>³²P-labeled DNA probe (20,000 cpm/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Proteins Extract</td>
<td>X μl</td>
</tr>
<tr>
<td>Sonication buffer</td>
<td>X μl</td>
</tr>
<tr>
<td>Total</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

A control reaction lacking purified proteins/extracts but containing all other components of the binding reaction was also set up. The reaction components were mixed gently and incubated at room temperature for 20-25 min. The samples were then loaded onto a 5% polyacrylamide gel. One extra lane with Bromophenol blue was also added so that the leading edge of the gel was visualised. The gels were first prerun for 20 min at 140 Volts and then for 1-1.5 hrs at 140 Volts.
2.30 Gel drying and autoradiography

After electrophoresis, the buffer was poured out of the electrophoresis tank and the plates disassembled. A piece of Whatmann 3 MM filter paper (cut to size) was placed on top of the gel, avoiding air bubbles and the paper lifted gently with the gel attached to it. This was then covered with cling film and placed in a vacuum gel dryer, with the gel facing up. The gel was dried at 80°C for 2 hrs. Once dry, the gel was placed in a cassette and exposed to X-ray film in the dark for at least 12 hrs at -80°C. The film was developed using a Xomat developing machine.

2.31 Protein determination using the Bicinchoninic acid assay (BCA)

♦ Preparation of standard curve as outlined by Smith et al. (1985)

This assay utilised the micro-plate protocol described in the Pierce kit insert. Fresh bovine serum albumin (BSA) was diluted from the stock (2 mg/ml). The diluent was the buffer in which the protein was assayed. Dilutions used were in the range of 2000-20 μg/ml.

♦ Preparation of the working reagent

A 1:50 dilution was made of the BCA working solutions B to A. Then, 25 μl of control (buffer used for blank) or sample was pipetted into the appropriate microwell. To this, 200 μl of WR was added. The solution was then shaken for 30 sec. The plate was covered and incubated at 37°C for 30 min. After incubation, the microwell plate was dried and allowed to cool at room temperature. The colour generated from the reaction was measured at 560 nm. Absorbance readings obtained for unknown concentrations of protein were determined from the standard curve. All
standards and unknown samples were assayed in triplicate. From the data obtained, a
standard curve was constructed, the equation of which, can be calculated and used to
determine the concentration of the protein content of the sample being investigated.

2.32 RNA extraction from bacterial cells

50 ml of culture was grown in LB and the cells were pelleted by centrifuging in a
microfuge at maximum speed for 5 min. The bacteria were then resuspended in
RNAWiz (1 ml RNAWiz for 2.5 OD_{600} U) by simply pipetting vigorously several
times. The samples homogenised in RNAWIZ can be stored at -20°C or -80°C for
up to a month. The homogenate was then incubated at room temperature for 5 min to
dissociate the nucleoproteins from the nucleic acids. Then, 0.2 X of the starting
volume of chloroform was added to the homogenate. The chloroform should not
contain isoamyl alcohol or other additives. The sample was covered and shaken
vigorously for approximately 20 sec and incubated at room temperature for 10
min. The mixture was then centrifuged at 10,000 rpm for 15 min at 4°C. The mixture
separated into 3 phases, the colourless upper aqueous phase (containing the RNA),
the semi-solid interphase (containing most of the DNA), and the lower organic
phase. Without disturbing the interphase, the aqueous phase was carefully
transferred into a clean RNase-free tube. 0.5 X of the starting volume of RNase free
water was added and the resulting volume mixed well. Then, 1X-starting volume of
isopropanol was added, well mixed and incubated at room temperature for 10 min.
The solution was then centrifuged at maximum speed for 15 min at 4°C to pellet the
RNA. The supernatant was discarded. The pellet was air dried for about 10 min. It is
important not to let the pellet dry completely as this will make it difficult to
resuspend. As well, it is not recommended to dry it under vacuum with
centrifugation. The RNA was then resuspended in an appropriate amount of RNase
free water (~150 µl/50 ml of culture). It was briefly subjected to vortex or
repeatedly pipetted to aid resuspension and if necessary heated to ~60°C.
2.33 RNA analysis by gel electrophoresis

In order to ascertain the integrity of RNA, isolated samples were run on 1.5% (w/v) agarose gels. The appropriate amount of agarose was dissolved in DEPC-treated H₂O and prepared according to the previous section. The RNA samples (1 μl) were prepared for electrophoresis by adding 3 μl of RNA sample buffer and made up to 15 μl in DEPC-treated H₂O. The samples were heated to 65°C for 10 min prior to loading on the gel. The gel was run in 1 X TAE. As ethidium bromide is included in the RNA sample buffer, the gels did not require further staining and could be visualised directly on a UV trans-illuminator.

2.34 Quantification of mRNA

The quantitation of mRNA for the measurement of gene expression was performed in a two-step procedure. In the first step, cDNA was prepared from RNA by reverse transcription using random hexamers as primers. During the second step, cDNA was amplified by real time PCR. Real-time PCR is increasingly being adopted for RNA quantification based on its ability to detect the amount of PCR product present at every cycle (i.e., in real time), as opposed to the endpoint detection by conventional PCR methods, thus allowing the real-time progress of the reaction, especially its exponential phase to be viewed. The real-time PCR approach is based on the detection and quantification of a fluorescent reporter, where the signal increases in direct proportion to the amount of PCR product in a reaction. SYBR green was the fluorescent reporter employed. SYBR green binds the double-stranded PCR product in a sequence-independent manner and will not bind single-stranded DNA (i.e., primers). The real-time system was used for comparative gene expression analysis, normalising with housekeeping genes.

Because PCR can even detect a single molecule of DNA, RNA samples were digested with Deoxyribonuclease I (DNasel) which is an endonuclease isolated from bovine pancreas that digests double and single-stranded DNA into oligo and
mononucleotides. This was necessary as no current RNA isolation procedure
removes 100% of the DNA.

♦ Preparation of RNA for RT-PCR

Ten-fold serial dilutions of total RNA were treated with Amplification Grade
DNasel according to the following procedure. The DNase-treated RNA and
untreated controls were assayed by RT-PCR. No loss in RT-PCR sensitivity was
detected with DNase-treatment, indicating that the kit components do not interfere
with RT-PCR and that they are free from significant RNase activity.

To an RNase-free PCR tube, was added

2 μg RNA sample diluted in DEPC H₂O 8 μl
10 X Reaction buffer 1 μl
Amplification Grade DNase I (1 U/μl) 1 μl

The reaction was then incubated for 15 min at room temperature. Then, 1 μl of stop
solution was added before heating to prevent metal (Mg/Ca) ion catalysed hydrolysis
of the RNA. Finally, the reaction was then chilled on ice for 5 min.

♦ Reverse transcription (RT)

This is the process whereby mRNA is transcribed into cDNA using a reverse
transcriptase, in this case Moloney Leukemia Virus reverse transcriptase (M-MLV
RT). Initially, 2 μl of random hexamers was added to 2 μg RNA and the volume
brought up to 10 μl with DEPC H₂O. The mixture was heated to 70°C for 5 min, to
destabilise secondary mRNA structures, and then placed on ice. Then, the reagents
listed below were added in the following order.
Reverse Transcriptase buffer (5 X) 8 µl
DNA mix (20 mM) 1 µl
MgCl₂ (25 mM) 4 µl
BSA (4 µg/µl) 1 µl
RNasin ribonuclease inhibitor 1 µl
M-MLV reverse transcriptase (200 U/µl) 2 µl
DEPC H₂O 13 µl

The reactions were placed in a Hybaid thermocycler at 37°C for 1 hr and 92°C for two min followed by storage at 4°C

♦ Real time PCR

12.5 µl of SYBR Green PCR Master mix containing Taq DNA polymerase, dNTPs, MgCl₂, and SYBR Green I dye was used. Each reaction with a total volume of 25 µl was set up as follows

cDNA 2 µl
SYBR Green 12.5 µl
Nuclease free H₂O 8.5 µl
Forward primer (0.4 µM) 1 µl
Reverse primer (0.4 µM) 1 µl

Samples were quantified using the Rotor Gene™ 3000 multiplex system (Corbett research) under the following thermo-cycling conditions
<table>
<thead>
<tr>
<th>Cycle Description</th>
<th>Temperature and Time Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First cycle (Denature)</strong></td>
<td>95°C for 15 min 1 cycle</td>
</tr>
<tr>
<td><strong>Second cycle (Cycling)</strong></td>
<td>95°C for 20 sec 50°C for 30 sec <strong>50 cycles</strong> 95°C for 30 sec</td>
</tr>
<tr>
<td><strong>Third cycle (hold)</strong></td>
<td>60°C for 1 min 1 cycle</td>
</tr>
<tr>
<td><strong>Final cycle (melt)</strong></td>
<td>50-99°C rising by 1°C each step, waiting for 15 sec on first step, then 5 sec for each step afterwards</td>
</tr>
</tbody>
</table>
2.35 Miller Assay

The method used was described by Miller (1972) A 1 ml aliquot of culture to be tested was centrifuged at 5,000 rpm in a microfuge tube for 5 min and the cell pellet was resuspended in 0.75 ml of Z buffer. The cells were permeabilised by the addition of 100 µl of chloroform and 50 µl of 0.1% SDS. The tube was vortexed for 10 sec and then equilibrated at 30°C for 5 min. The reaction was started by the addition of 0.2 ml of ONPG (4 mg/ml in Z buffer, prepared fresh) and the tube was vortexed again for 10 sec. The reaction was timed for 10-20 min and then stopped by the addition of 0.375 ml of 1 M Na₂CO₃. The cells were then pelleted by centrifugation at 13,000 rpm in a microfuge tube and OD₄₂₀ of the supernatant recorded. β-galactosidase activity was calculated using the equation below:

\[
\text{Miller units} = \frac{\text{OD}_{420} \times 1000}{V \times T \times \text{OD}_{600}}
\]

- OD₄₂₀ = the absorbance of the supernatant at 420 nm
- OD₆₀₀ = the absorbance of the culture at 600 nm
- V = the volume of the culture used in ml
- T = the time of the reaction in min

2.36 GFP-UV expression

Qualitative green fluorescent protein-ultraviolet (GFP-UV) expression of cultures grown on TY broth was evaluated by visualisation of cultures under bright and UV light using a microscope 100X objective with oil. For quantitative measurements of fluorescence of GFP-UV in cultures, cultures were grown in TY broth medium supplemented with gentamicin 15 µg/ml and with 2,2'-dipyridyl if under iron deplete conditions (250 µM for S. meliloti 2011rhrA26 and 300 µM for S. meliloti 2011 and 2011rhrA2). When the culture reached late exponential phase, 100 µl was
transferred to microtiter plates (three cultures were grown per condition and readings were done in triplicate), and fluorescence was evaluated with a luminescence spectrometer LB 50 using a 490 nm excitation and 520 nm emission. Cell optical density at 600 nm was measured. Quantitative fluorescence was determined according to Tang et al. (1999)

2.37 Iron nutrition bioassays to detect siderophore utilisation

Siderophore utilisation bioassays (O Cuiv, 2003) were performed in media prepared with ultra pure water and supplemented with the appropriate concentration of 2,2' dipyridyl. Molten agar (1.5% with Oxoid N°1 purified technical agar) prepared in 25 ml aliquots, was inoculated with 200 µl of stationary phase culture and the appropriate concentration of 2,2' dipyridyl usually 300 µM for S. meliloti, and the mixtures were poured into sterile plates. Wells were cut out of the solid media, and 50 µl of the test solutions were pipetted into the wells. Growth was allowed to proceed for 24 to 48 hours, and plates were then examined for haloes of bacterial growth surrounding wells bearing test solutions.

Test solutions (concentrated culture supernatants) were prepared by adding 2,2' dipyridyl to the appropriate concentration to broth, usually 300 µM for S. meliloti, and then inoculating with the relevant strain. Growth was allowed to proceed until late log phase. The culture was transferred into 1.5 ml aliquots to microfuge tubes and centrifuged at 13,000 rpm for 3 mm to pellet the cells. Cell free supernatants were transferred to fresh tubes and concentrated in a vacuum dryer set to high temperature, and then resuspended in one-tenth the original volume with ultra pure water. The samples were then pooled, filter sterilised through a 0.45 µm filter and stored in the dark at -20°C.
2.38 Molecular maker used for the different reactions

1Kb ladder used for southern blot and DNA agarose gels and protein standard markers used for SDS-PAGE gels.
Chapter 3:
Identification and characterisation of the *fur* gene in *Sinorhizobium meliloti*
3.1 Introduction

The availability of iron to rhizobia free-living in the soil is potentially limiting due to the insolubility of ferric iron and also because rhizobia have to compete with other microorganisms to acquire the available iron. *S. meliloti* 2011 produces one known siderophore, rhizobactin 1021 that has been shown to be inessential for symbiotic nitrogen fixation (Lynch et al., 2001). It is likely to contribute to the competitiveness of the bacterium when free-living in the soil. Furthermore, rhizobia in symbiosis display a high requirement for iron, as many of the proteins involved in nitrogen fixation require the metal as a cofactor.

In many gram-negative bacteria, the ferric uptake regulator (Fur) protein controls the production of siderophores playing a central role in the control of genes involved in iron homeostasis. Because iron is an important metal in the agriculturally important symbiosis between alfalfa and its nitrogen-fixing endosymbiont *S. meliloti*, the role of Fur was investigated in the organism. The aim of the investigation was to understand the role of Fur in the regulation of iron acquisition systems, including rhizobactin 1021 in the context of maintaining an overall balance of iron within the cell.

Analysis of the rhizobactin operon has revealed the presence of 10 ORF’s which have been shown to be or to have a high probability of being, functional genes. The characterization of the ORF’s was undertaken by mutation and by bioinformatic analysis. Six of the ORF’s showed homology to siderophore biosynthesis genes and were designated *rhbA*, *B*, *C*, *D*, *E*, and *F* respectively. The protein products of two further ORF’s showed homology to an AraC-like transcriptional regulator and to a siderophore outer membrane receptor and were designated *rhrA* and *rhtA* respectively (Lynch et al., 2001 and PhD Thesis, 1999). The protein product of the ninth ORF, designated *rhbG*, showed homology to siderophore biosynthesis proteins but as yet, no function as been assigned to it. The final ORF was recently
characterized as a permease and named \textit{rhtX} (Ó Cuív \textit{et al.}, 2004). Fig 3.1 shows the positions and orientations of the above mentioned genes.

\begin{center}
\begin{tikzpicture}
  \node at (0,0) (rhtX) {\textbf{rhtX}}; 
  \node at (1,0) (rhbA) {\textbf{rhbA}}; 
  \node at (2,0) (rhbB) {\textbf{rhbB}}; 
  \node at (3,0) (rhbC) {\textbf{rhbC}}; 
  \node at (4,0) (rhbD) {\textbf{rhbD}}; 
  \node at (5,0) (rhbE) {\textbf{rhbE}}; 
  \node at (6,0) (rhbF) {\textbf{rhbF}}; 
  \node at (7,0) (rhrA) {\textbf{rhrA}}; 
  \node at (8,0) (rhlA) {\textbf{rhlA}}; 
  \node at (9,0) (rhbG) {\textbf{rhbG}}; 

  \foreach \i in {0,...,9} { 
    \draw[->,thick] (\i,-0.2) -- (\i+1,-0.2); 
  }

  \draw[->,thick] (rhtX) -- (rhbA) node[above,midway] {\textbf{Permease}}; 
  \draw[->,thick] (rhbA) -- (rhbB) node[above,midway] {\textbf{Siderophore Biosynthesis}}; 
  \draw[->,thick] (rhbC) -- (rhbD) node[above,midway] {\textbf{AraC type regulator}}; 
  \draw[->,thick] (rhbD) -- (rhbE) node[above,midway] {\textbf{Outer membrane receptor}}; 

  \draw[->,thick,orange] (rhbF) -- (rhrA) node[above,midway,orange] {\textbf{Outer membrane receptor}}; 
  \draw[->,thick,orange] (rhrA) -- (rhlA); 
  \draw[->,thick,orange] (rhlA) -- (rhbG) node[above,midway,orange] {\textbf{Outer membrane receptor}}; 

\end{tikzpicture}
\end{center}

\textbf{Legend:}
- Permease
- Siderophore Biosynthesis
- AraC type regulator
- Outer membrane receptor

\textbf{Fig 3.1: Organisation of the rhizobactin 1021 regulon}

Fur in \textit{E. coli} binds under iron replete conditions to the promoter regions of the regulated genes on an operator sequence called the 'Fur box'. It was decided to search the genome of \textit{S. meliloti} in order to identify the Fur homologue. The gene was then cloned into an expression vector to overexpress and purify the protein. Its functionality was checked by assessing its complementation of an \textit{E. coli fur} mutant. The protein was overproduced with the aim of characterising the promoter regions bound by the regulator by the electrophoretic mobility shift assay (EMSA).
3.2 Identification of the Fur homologue in S. meliloti

3.2.1 Blast analysis

*Smc02510* was identified as the *S. meliloti* Fur homologue by BlastX analysis using the NCBI database. The protein encoded by *Smc02510* is 42% identical to the one encoded by *E. coli* K12 *fur* and was therefore the primary candidate to be the functional Fur homologue (Fig. 3.2).

![Amino acid sequence alignments of Fur from *S. meliloti* 2011 (*Smc02510*) and Fur from *E. coli* K12.](image)

**Fig 3.2. Amino acid sequence alignments of Fur from *S. meliloti* 2011 (*Smc02510*) and Fur from *E. coli* K12.**

Recently, several Fur-like proteins have been identified that are not functional Fur homologues, but instead are involved in the maintenance of zinc homeostasis (Gaballa et al., 1998, Patzer et al., 1998), manganese-dependent response to oxidative stress (Bsat et al., 1998) or iron-dependent regulation of haem biosynthesis (Hamza et al., 1998). Additional fur-like genes have been identified from genome sequencing and from screens for genes involved in pathogenesis (Camilli et al., 1995, Wang et al., 1996). There now appears to be a family of Fur proteins that are functionally diverse, but are all involved in metal-dependent regulation. As a consequence, it was not unexpected to obtain more than one Fur candidate from the Blast analysis.

From the *S. meliloti* genome, two additional proteins were also identified as Fur-like proteins.
• With 28% identity: Smc00329, which is a homologue of Irr (Iron response regulator) in *B. japonicum*. Irr regulates haem (Hamze *et al.*, 1998). Identified and characterised in *B. japonicum*, this protein may be the most divergent of the Fur-like proteins described so far in that it is only active under metal limitation and contains a single cysteine residue rather than the multiple cysteines found in the other proteins. Moreover, *irr* gene expression is strongly regulated by iron whereas *fur* is essentially constitutive.

• With 31% identity: Smc04242, which encodes Zur, which is a putative zinc uptake regulator.

### 3.2.2 Smc02510: The primary fur homologue in *S. meliloti*

*Smc02510* is a 429 bp gene present as a single copy on the chromosome (Fig 3.3). Located downstream from the *fur* gene, an ABC transporter system encoded by the *sitABCD* operon is present and was characterised by Platero *et al.* (2003) as a manganese transport system.

![Diagram of chromosomal location](image)

**Fig 3.3: Chromosomal location of fur**

In the intergenic region between *fur* and *sitABCD* a putative ‘Fur box’ was identified (Fig. 3.4).
3.3 Cloning of *S. meliloti fur*.

The development of recombinant DNA technology has made feasible the overexpression of proteins in *E. coli*. However, each gene presents unique challenges for its overproduction and it is often necessary to optimise the regulatory elements and growth conditions for high-level expression. Different vectors are available with a variety of features.

pQE, a series of commercial vectors with prominent advantages have been widely used for overexpression of proteins in the cytoplasm of *E. coli*. They contain a powerful expression cassette composed of a phage T5 promoter, two lac operator sequences, a synthetic ribosome binding site (RBS), and an optimised codon sequence MRGSH6GS at the N-terminus of the target protein to improve expression up to as much as 50% of total cellular protein (Fig 3.5). It was decided to use pQE60 from Qiagen that would allow the overexpression of the Fur protein with a His tag fused to its C-terminal.
The *fur* gene from *S. meliloti* 2011 was amplified from *S. meliloti* genomic DNA by PCR. The restriction sites *Ncol* and *BamHI* sites were incorporated into the forward and reverse primers respectively having the following sequences:

- **Fur60-F**:
  
  CCATGG AGAGCCAGAGCAAGAATCGGATCG

- **Fur60-R**:
  
  GGATCC GTC CTTGCGCTTCCGGCAATAG

The ATG in the restriction site of *Ncol* was used as the start codon for Fur. The amplified fragment extends from the start codon to the final codon before the stop codon allowing a 6 histidine tag to be added to the C-terminus of the recombinant Fur (Fig 3.6). The cloning strategy is outlined in Fig 3.7. This 438-bp fragment generated by PCR was cloned into the pCR2.1 vector. The *Ncol-BamHI* fragment carrying the PCR-generated product was subcloned into the expression vector pQE60. pQE60 is a high copy number plasmid that allows high-level regulated expression of C-terminal 6xHis-tagged proteins in *E. coli*.
POE-60

Fig 3.6: Cloning of the *fur* gene into the multiple cloning site of pQE60
Fig 3.7: pFur60 Cloning strategy
The Polymerase Chain Reaction (PCR) program used is described in the Table 3.1.

**Table 3.1: PCR Reaction Conditions for the amplification of the *S. meliloti fur* gene.**

<table>
<thead>
<tr>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealing Temperature 66°C</td>
</tr>
<tr>
<td>Annealing Time 1 min</td>
</tr>
<tr>
<td>Extension Time 72°C for 1 min</td>
</tr>
</tbody>
</table>

Following the cloning, the resulting vector, designated pFur60, was transformed into *E. coli* XL10 gold for overexpression and purification and *E. coli* H1681 to check the functionality of the recombinant protein and to assess *E. coli fur* complementation.
3.4 Complementation of an *E. coli* fur mutant

Many Gram-negative bacterial species possess a *fur* system with close enough homology to allow the complementation of a *fur* mutation in *E. coli* (Litwin et al., 1992, Wooldrige et al., 1994, Yamamoto et al., 1997 and Bereswill et al., 1998). The complementation assay was used to determine if the recombinant protein *S. meliloti* Fur was functionally active despite the presence of the His-tag and also if the protein functions in a similar way to *E. coli* Fur. The importance of controlling iron intake has led to the conservation of *fur* regulation in a wide spectrum of bacteria.

In order to discover whether or not, Fur from *S. meliloti* binds to the canonical Fur box, a Fur complementation on an *E. coli* *fur* mutant was thus performed.

For the complementation assay, *E. coli* H1681 carrying a mutation in the *fur* gene was used. It also possesses the *lac* gene under the control of the promoter of the *fur*-regulated *fhuF* gene encoding a ferric hydroxamate uptake protein. This promoter contains the canonical ‘Fur box’.
3.4.1 Principle of the complementation assay

**McConkey Agar +2,2'-D**

**McConkey Agar +FeCl₃**

Fig 3.8: Principle of the complementation assay

The principle of the assay is that:

- The strain used carries a mutation in the endogenous *fur* gene and relies on an introduced *fur* gene for Fur activity.
- **Under iron deplete conditions**, i.e. in the presence of 2,2'-dipyridyl, no ferrous iron is available to act as a cofactor for Fur and thus the repressor cannot bind to the promoter region of the *E. coli fhuF* gene giving rise to red colonies (Fig 3.8).

- However, **under iron replete conditions**, the ferrous iron can bind to the transcriptional repressor. If it is functional, the dimer can then bind to the *fhuF* promoter region, giving rise to white colonies or in some cases, if the complementation is only partial, to pink colonies (Fig 3.8).
3.4.2 Results

*E. coli* H1681 bearing either pFur60 or pQE60 (the empty vector as a negative control) were plated onto ampicillin MacConkey agar containing either 200 μM 2,2'-dipyridyl or 0.1 mM FeCl₃ and incubated overnight at 37°C.

The plates showed that under iron replete conditions, the induction of pFur60 resulted in the production of *S. meliloti* Fur that had bound to the *E. coli fhuF* promoter giving rise to pink colonies (Fig 3.9, 3.10 and Table 3.2).

**Table 3.2: Complementation Assay results**

<table>
<thead>
<tr>
<th>Strain</th>
<th>McConkey Agar + FeCl₃</th>
<th>McConkey Agar + 2,2'-dipyridyl</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> H1681 + pFur60</td>
<td>Pink</td>
<td>Red</td>
</tr>
<tr>
<td><em>E. coli</em> H1681 + pQE60</td>
<td>Red</td>
<td>Red</td>
</tr>
</tbody>
</table>
Fig 3.9: Fur complementation on Mc Conkey Agar supplemented with FeCl₃ (iron replete conditions).
On the left, colonies from a culture of *E. coli* H1681 containing pQE60 induced with IPTG at a concentration of 0.1 mM for four hrs; on the right, colonies from a culture of *E. coli* H1681 containing pFur60 induced with IPTG at a concentration of 0.1 mM for four hrs.

Fig 3.10: Fur complementation on Mc Conkey Agar supplemented with 2,2'-dipyridyl (iron deplete conditions).
On the left, colonies from a culture of *E. coli* H1681 containing pQE60 induced with IPTG at a concentration of 0.1 mM for four hrs; on the right, colonies from a culture of *E. coli* H1681 containing pFur60 induced with IPTG at a concentration of 0.1 mM for four hrs.
Therefore, the complementation showed that the recombinant *S. meliloti* Fur with the His-tag is a functional protein. The protein was able to bind partially to the promoter region of the *E. coli* *fhuF* gene.

### 3.5. Overexpression of *S. meliloti* Fur in *E. coli* XL10 gold.

In order to perform the mobility shift assay to investigate the physical interaction between *S. meliloti* Fur and the promoters it regulates, the regulator had to be produced in enough quantity that a band shift could be detected.

To overexpress the transcriptional regulator from pFur60, an *E. coli* strain harboring a lacI\(^q\) mutation is desirable. For the following work, it was decided to use *E. coli* XL10 gold.

A series of different expression conditions were assessed to optimise the recombinant protein induction and to obtain the highest possible yield of the protein.

A time course was undertaken to determine the optimum length of time for culture growth at 37°C after induction with IPTG. Gradients of different lengths of sonication and different concentrations of IPTG were used to determine the optimal conditions. In each case, the optimisation was carried out under native and denaturing conditions. Indeed, often the amount of native proteins lost under certain conditions, due for example to the formation of inclusion bodies, can be appreciated by comparing the amount of recombinant protein detected under native and denaturing conditions.

#### 3.5.1 Optimisation of the time of induction

To optimise the expression of the recombinant Fur, a time-course analysis of the level of protein expression following induction was carried out. This was done on a
small scale (culture volume). The use of small-scale expression cultures provides a rapid way to judge the effects of varied growth conditions on expression levels and the solubility of recombinant proteins. Induction was undertaken with IPTG at 0.1 mM, which was optimised as described below.

The level of expression of *S. meliloti* Fur over a period of six hrs post induction was analysed to determine the optimum time post induction for culture growth. Proteins were prepared under denaturing and native conditions and analysed by SDS-PAGE (Fig 3.11 and Fig 3.12).

Fig 3.11: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: Time course of expression of Fur under denaturing conditions.

Lane 1: Ladder
Lane 2: Non induced at time 0
Lane 3: Induced at time 0
Lane 4: Non induced after 1 hr
Lane 5: Induced after 1 hr
Lane 6: Non induced after 2 hrs
Lane 7: Induced after 2 hrs
Lane 8: Non induced at time after 4 hrs
Lane 9: Induced after 4 hrs
Lane 10: Non induced at time after 6 hrs
Lane 11: Induced after 6 hrs
It was decided that a time of four hrs growth post induction was giving the best results under native conditions. As well, there is not a noticeable difference between the yield of proteins obtained under native and denaturing conditions, which would suggest that the *S. meliloti* Fur is a stable protein and is not subject to the formation of inclusion bodies.

### 3.5.2 Optimisation of the concentration of IPTG

The expression of the recombinant Fur was induced with IPTG. However, the inducer can present disadvantages, one of which is its toxicity to the cell. IPTG does influence *E. coli* metabolism substantially, altering both the synthesis of certain
proteins and the specific growth rate (Kosinski et al., 1992). Indeed, a strong IPTG-induced expression of recombinant genes often inhibits cellular growth. This growth inhibition is suggested to be caused by a perturbed balance of protein synthesis after induction. The strong increase of induced mRNA affects general cellular maintenance by causing a reduced synthesis of proteins necessary for growth and reproduction (Vind et al., 1993; Dong et al., 1995; Rinas, 1996). Dong et al. (1995) have shown the rapid inhibition of ribosomal RNA synthesis, and even the degradation of ribosomes after a strong induction by IPTG.

It is thus important to limit the concentration of inducer used to start the expression of the recombinant proteins to the minimum necessary. Protein yields obtained four hrs post induction, from a gradient of 0.05 to 1 mM IPTG for the induction of the culture, were compared under native and denaturing conditions (Fig 3.13 and Fig 3.14).

![Fig 3.13: SDS-PAGE gel loaded with protein preparations from E. coli XL10 Gold, pFUR60: Optimisation of the concentration of IPTG for mini prep under denaturing conditions.](Lane 1: Ladder)
Fig 3.14: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: Optimisation of the concentration of IPTG for mini prep under native conditions.

Lane 1: Ladder
Lane 2: Non-induced
Lane 3: Induced with IPTG at a concentration of 0.05 mM
Lane 4: Induced with IPTG at a concentration of 0.10 mM
Lane 5: Induced with IPTG at a concentration of 0.25 mM
Lane 6: Induced with IPTG at a concentration of 0.50 mM
Lane 7: Induced with IPTG at a concentration of 1.00 mM

The results showed no detectable difference in protein levels following induction with the different concentrations of IPTG. While a concentration of 0.05 mM IPTG was shown to be sufficient for induction, it was decided to use 0.1 mM in
subsequent experiments. This concentration is well below that which has been shown to cause toxicity.

3.5.3 Optimisation of the time of sonication

Finally, the last optimisation was for the sonication time used to lyse the cells during protein preparation. Sonication for too long under native conditions would perturb the quaternary structure of the protein while on the other hand, it has to be long enough to break down the *E. coli* envelope to release recombinant proteins from the cytoplasm.

The sonication was performed using a 3 mm micro-tip sonicator (Sonics & Materials Inc.) using 2.0 sec, 40 kHz pulses. Different times of sonication were applied to the bacterial cells, which were carefully kept on ice and the results compared (Fig 3.15 and 3.16).
Fig 3.15: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: Optimisation of the time of sonication for mini preps under native conditions. Samples were induced with 0.1 mM IPTG.

Lane 1: Ladder
Lane 2: No sonication
Lane 3: 20 s sonication time
Lane 4: 40 s sonication time
Lane 5: 60 s sonication time
Lane 6: 80 s sonication time
Lane 7: 100 s sonication time
Fig 3.16: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: Optimisation of the time of sonication for mini preps under denaturing conditions.

Lane 1: Ladder
Lane 2: No sonication
Lane 3: 20 s sonication time
Lane 4: 40 s sonication time
Lane 5: 60 s sonication time
Lane 6: 80 s sonication time
Lane 7: 100 s sonication time

While 20 seconds is sufficient under denaturing conditions, 40 seconds is the minimal length of sonication time necessary to extract the native recombinant *S. meliloti* Fur.
As the goal is to purify a large amount of recombinant protein, Fur was also produced in large scale (100 ml) and the sonication had to be optimised for such a volume.
Fig 3.17: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: Optimisation of the time of sonication for large preps under native conditions

Lane 1: Ladder
Lane 2: Protein prep induced with 0 s for sonication time
Lane 3: Protein prep induced with 30 s for sonication time
Lane 4: Protein prep induced with 60 s for sonication time
Lane 5: Protein prep induced with 90 s for sonication time
Lane 6: Protein prep induced with 120 s for sonication time

A sonication of 1 min is necessary to extract *S. meliloti* Fur from large scale cultures.
3.5.4 Conclusion

Following the optimisation procedures described for length of growth period after IPTG induction, IPTG concentration used for induction and sonication, it was concluded that induction with 0.1 mM IPTG followed by growth for 4 hrs was optimum. For small scale studies using 1.5 ml cultures, 40 seconds sonication was used while 1 min sonication was used for 100 ml cultures.

After optimisation of the overexpression, it can be said using a density program that the native *S. meliloti* recombinant Fur represents about 15% of the total protein content of *E. coli* XL10 gold, pFur60 (Fig 3.18).

![Fig 3.18: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: *S. meliloti* recombinant Fur expressed under native conditions](image)

Lane 1: ladder  
Lane 2: Non induced culture  
Lane 3: Induced culture
3.6 Purification of the Fur protein by IMAC (Immobilised metal affinity chromatography)

3.6.1 Principle of IMAC

IMAC involves the affinity binding of His-tagged proteins to the nickel ions immobilized on a matrix. The imidazole ring is the part of the histidine structure, which binds to the nickel ions immobilized by the matrix. Therefore, imidazole itself can also bind to the nickel ions and disrupt the binding of histidine residues, thus releasing a tagged protein. One major consideration in the purification of proteins is the concentration of imidazole used (Fig 3.19).

![Chemical structures of histidine and imidazole](image)

**Fig 3.19 Chemical structures of histidine and imidazole**

Since the *S. mehlotti* recombinant Fur was intended for use in mobility shift assays and so was needed in the native state, the recombinant protein was purified from an *E. coli* protein extract in which the protein was released from the cells under native conditions.
There is no general protocol for purifying a protein under native conditions, as each protein has different requirements. However, some general suggestions found in the literature helped to optimise the native purification of *S. meliloti* Fur (Makrides et al., 1996).

### 3.6.2 Optimisation of the buffers for IMAC

All buffers should have sufficient ionic strength to prevent nonspecific interactions between proteins and the resin and so a salt concentration of 300 mM NaCl was used in the sonication, wash, and elution buffers.

Because a low concentration of imidazole in the lysis and wash buffers minimise non-specific binding and reduces the amount of contaminating proteins, 10 mM imidazole was added to the sonication buffer. For the washing buffers a higher concentration had to be added. To determine the appropriate concentration of imidazole, different concentrations were added to the protein extracts to determine the highest concentration of imidazole that can be applied to the column without precipitating the recombinant protein (Fig 3.20).
Fig 3.20: 15 % SDS polyacrylamide gel. Native protein preparations from *E. coli* XL10 Gold, pFur60 analysed by addition of washing buffers with a gradient of imidazole concentrations.

Lane 1: Ladder  
Lane 2: 5 mM Imidazole  
Lane 3: 10 mM Imidazole  
Lane 4: 25 mM Imidazole  
Lane 5: 50 mM Imidazole  
Lane 6: 75 mM Imidazole  
Lane 7: 100 mM Imidazole  
Lane 8: 150 mM Imidazole  
Lane 9: 200 mM Imidazole  
Lane 10: 225 mM Imidazole  
Lane 11: 250 mM Imidazole

A concentration of 150 mM imidazole was chosen, as it does not precipitate the recombinant Fur while competing with a lot of the non-specific proteins that have bound to the resin.

Finally, 250 mM imidazole was the concentration of imidazole chosen to precipitate the recombinant protein.
3.6.3 Optimised purification protocol

After sonication of the bacterial cells in lysis buffer, 5 ml (250 μl for a small scale preparation) of the resulting solution was added to a universal bottle containing 1 ml (100 μl for a small scale preparation) of an IDA metal resin (Invitrogen) charged with nickel. Binding of the protein resulted from the binding of the 6xHis-tag attached to Fur to the nickel. This was promoted by shaking the universal at 4°C for one hr. This step promotes the efficient binding of the His-tagged recombinant protein especially in case the His-tag is not fully accessible or if the concentration of Fur in the lysate is low. Then, the resin was washed twice with 5 ml (200 μl for a small scale preparation) of the washing buffer, containing 150 mM imidazole, and finally eluted three times with 2.5 ml (50 μl for a small scale preparation) of the elution buffer, containing 250 mM imidazole. The eluted proteins were pooled together.

An example of the results of a recombinant *S. meliloti* Fur purification carried out in large scale is shown in Fig 3.21.
Fig 3.21: 15 % SDS polyacrylamide gel following purification of native Fur protein and IMAC purification of recombinant His-tagged-Fur.

Lane 1: Ladder
Lane 2: Fur native preparation
Lane 3: Wash through
Lane 4: Wash 1 with 150 mM Imidazole
Lane 5: Wash 2 with 150 mM Imidazole
Lane 6: Elution 1 with 250 mM Imidazole
Lane 7: Elution 2 with 250 mM Imidazole
Lane 8: Elution 3 with 250 mM Imidazole

Following the purification, a Dialysis was then performed to remove the imidazole as it could affect the performance of the mobility shift assay. *S. meliloti* Fur was dialysed overnight at 4°C against 20 mM Tris/HCl pH 8.0 and stored at -20 °C. Purified Fur was prepared in this way for the mobility gel shift assays.
3.7 Electrophoretic Mobility Shift Assay (EMSA)

The mobility shift assays were performed with the aim of determining the binding activities of the purified recombinant Fur from *S. meliloti* to different *S. meliloti* promoters known to be iron responsive. The analysis of the transcriptional regulator was mainly concentrated on investigating its role in the regulation of the genes involved in rhizobactin 1021 mediated iron uptake.

The DNA probes for the mobility shift assay were prepared by PCR and the regions amplified are indicated in Fig 3.22.

![DNA probes for mobility shift assay](image)

**Fig 3.22: DNA probes for mobility shift assay.**
The amplified regions are shown (→) for the *fhuF* promoter region of *E. coli*, a positive control (1), iron responsive promoters from *S. meliloti* (2, 3 and 4) and the promoter region of the *fur* gene in *S. meliloti* (5).

The promoter region of *fhuF* was amplified to be used as a positive control. From the complementation described in section 3.4.2, it is known that *S. meliloti* Fur binds the *fhuF* promoter and thus this mobility shift assay would demonstrate that the experimental conditions are correct.

RNase Protection Assays (RPA) carried out previously (Lynch *et al.*, 2001) have shown that the operon *rhtXrhhABCDEF* which encodes the rhizobactin 1021 permease and biosynthesis genes, are iron responsive. Thus, the binding of Fur to the
promoter of this operon was investigated. Also, the outer membrane receptor encoded by \textit{rhtA} is known to be iron responsive and therefore the intergenic region between \textit{rhrA} and \textit{rhtA} was also investigated for Fur binding.

Also, the promoter region of \textit{smc02726}, a gene encoding the outer membrane receptor for haem utilisation in \textit{S. meliloti}, which was characterised by another member of the research group (Paraic O Cuiv, unpublished data) was investigated.

Finally, regulators are found to be autoregulatory in many cases. In \textit{E. coli}, Fur expression is constitutive. However, \textit{E. coli} Fur can bind weakly to its own promoter and downregulate its expression. Also, the regulator often regulates adjacent genes. Thus, the intergenic region of \textit{fur-sitABCD} was amplified to be used as a probe.
The double stranded oligonucleotide probes were amplified by PCR and labelled as described in chapter 2. Specific primers as shown below were designed to amplify different promoter regions of *E. coli* H1681 and *S. meliloti* 2011. After amplification from genomic DNA, the PCR products were purified and visualised by agarose gel electrophoresis. The resulting PCR products range from 100 to 250 bp:

**Promoter sequence upstream *flhuF***:

MSAfhuF-F:

`CGGGATCC CGG AAC GAT AGG CCA TAA TCG GG`

MSAfhuF-R:

`CGGGATCC TCC CCA GCC ACT GCC CAG CG`

```
CGGGATCCGGAACGATAGGCCATAATCGGGATAGTAATCTAAATGATAATGATTGCTAATCATAGCGATAGGTTTACCCGATAGCAAGGGATTATCTGGCTTGCAAATGATAAAAATTATCATATGATATTGGTTATCAATGACCAGCATACATGTTGCAACGTACGCTGGGCAGTGGCTGGGGAGGATCCCG
```

Probe length: 205bp. Highlighted in orange is the *E. coli* Fur Box and purple the *BamHI* sites used in labelling the probe.

**Promoter sequence upstream *rhtX***:

MSARHTX-F:

`CGGGATCC CCT ATC GCC TCT CTC GAA AAT GC`

MSARHTX-R:

`CGGGATCC CGA AAA CTG CCA CTG CCC GGC`

```
CGGGATCCCGGAACGATAGGCCATAATCGGGATAGTAATCTAAATGATAATGATTGCTAATCATAGCGATAGGTTTACCCGATAGCAAGGGATTATCTGGCTTGCAAATGATAAAAATTATCATATGATATTGGTTATCAATGACCAGCATACATGTTGCAACGTACGCTGGGCAGTGGCTGGGGAGGATCCCG
```

Probe length: 160 bp. Highlighted in orange is the *E. coli* Fur Box and purple the *BamHI* sites used in labelling the probe.

**Intergenic sequence between *rhrA* and *rhtA***:

MSArhra-F:

`CGGGATCC GTC GTG CGC CAG CCT TTC CTG`

```
CGGGATCCCTATCGCCTCTCGAAGATGCTGTTGGCTACTGCAGATTACATGATGATTGGCTATTAAAGATGACCAGCATACATGGTGCAACGTACGCTGGGCAGTGGCTGGGGAGGATCCCG
```

Probe length: 160 bp. Highlighted in orange is the *E. coli* Fur Box and purple the *BamHI* sites used in labelling the probe.
MSArhra-R:

CGGGATCC T GCC CAT AA CGC CCC CTG CGC

CGGGATCCGTCGTGCGCCAGCCTTTCCTGTTGACGTTCGCATGCGTC
CAAATGAGGTTCGCCATTATCCAAGCGGCGAACACCCTTAGCCCATA
AAACATGACTTAAATAGTCTTGTATTGGCAATTTGCCCGCCCACCGG
CAGCGGCAATTGTTTTTCTGTTGCGCAGGGGCGTTATGGGCAGGAT
CCCG

Probe length: 191 bp. Highlighted in orange is the *E. coli* Fur Box and purple the *BamHI* sites used in labelling the probe.

Promoter sequence *smc02726*:

MSAheme-F:

CGGGATCC GGA CCA GTC CTT TGA AAG TGT TGG

MSAheme-R:

CGGGATCC GTT TTC TTA TGT GAC GAA AAT AAG GC

CGGGATCCGGACCAGTCCCTTTGAAAAGTGTTGGCGGGGGCTTGCTTT
GAGCGGCGAATCCAAGGGCCTGGGCGAGTGGGAAATGGGCGAAG
GCCATCTATTTGGGGCTTTATTTTCGTCACATAAGAAGACCGGATCCCG

Probe length: 139 bp. Highlighted in orange is the *E. coli* Fur Box and purple the *BamHI* sites used in labelling the probe.

Intergenic sequence between *fur* and *sitA*:

MSAsitA-F:

CGGGATCC CCC GCG ACA CTA GCC AAG GGG

MSAsitA-R:

CGGGATCC CCG GCT CTC CTC TTT GCG AAC C

CGGGATCCCCCGGCGACACTAGCGCAAGGGGACACCTTTTTGGAATAG
CTAGTTGCAAATGCTTTCTCAATTGGCATGACTCATGCAAGACCATTGC
CCTACCCATATTATGCTCGCAAAGAGGAGAGCCCGGGGATCCCG

Probe length: 137 bp. Highlighted in orange is the *E. coli* Fur Box and purple the *BamHI* sites used in labelling the probe.

The PCR program used is described in the Table 3.3.
Table 3.3: PCR Reaction Conditions for the amplification of the different probes.

<table>
<thead>
<tr>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealing Temperature 64°C</td>
</tr>
<tr>
<td>Annealing Time 1 min</td>
</tr>
<tr>
<td>Extension Time 72°C for 1 min</td>
</tr>
</tbody>
</table>

Cell extracts for mobility shift assays

In addition to using purified Fur, EMSAs were also conducted with cell extracts containing overexpressed Fur.

Cell extracts were prepared from *E.coli* XL10 Gold carrying the following plasmids:

- Bearing the vector pQE60 and which was induced for 4 hrs with a concentration of IPTG of 0.1 mM. This was used as the negative control instead of a non-induced culture of pFur60 as the latter could lead to a leaky expression of the protein.

- Bearing the vector pFUR60 and which was induced for 4 hrs with a concentration of IPTG of 0.1 mM. In this sample, the recombinant *S. meliloti* Fur is overexpressed.
3.7.1 EMSA with purified Fur

To check the conditions of the experiment and confirm the previous results, that *S. meliloti* Fur binds to the promoter region of *E. coli* *fhuF*, different concentrations of purified Fur were mixed with *fhuF* promoter region probes (Fig 3.23).

![Probes](image)

**Fig 3.23: EMSA with purified Fur and the promoter region of *fhuF***

Lane 1: Negative control containing no protein but only binding buffer  
Lane 2: 15 μM of Fur with binding buffer  
Lane 3: 30 μM of Fur with binding buffer  
Lane 4: 75 μM of Fur with binding buffer  
Lane 5: 150μM of Fur with binding buffer

A band shift was observed with as little as 15 μM of purified Fur. The protein concentrations were calculated using the BCA assay method as described in chapter 2. *S. meliloti* Fur bound to the probe confirming that the mobility shift assay is performed under the right conditions and that the recombinant protein can bind to an *E. coli* ‘Fur box’. Thus, *S. meliloti* Fur functions heterologously in *E. coli* as a ferric uptake regulator.
Given the evidence that the purified *S. meliloti* Fur was capable of binding a Fur box, its action was tested on several promoter regions of *S. meliloti* genes that are expressed in an iron-regulated fashion.

The DNA binding activity of *S. meliloti* Fur was investigated using a DNA fragment containing the promoter region of *rhtXrhhABCDEF* (Fig 3.27), the promoter region of the heme receptor *smc02726* (Fig 3.24), the intergenic region *rhrA-rhtA* (Fig 3.25), and finally the intergenic region (*fur-sitA*) (Fig 3.26).

Fig 3.24: EMSA with purified Fur and the region from the heme receptor *smc02726*

- **Lane 1**: Negative control containing no protein, only binding buffer
- **Lane 2**: 15 μM of Fur with binding buffer
- **Lane 3**: 30 μM of Fur with binding buffer
- **Lane 4**: 75 μM of Fur with binding buffer
- **Lane 5**: 150 μM of Fur with binding buffer
Fig 3.25: EMSA with purified Fur and the intergenic region between *rhrA* and *rhtA*

Lane 1: Negative control containing no protein but only binding buffer
Lane 2: 15 μM of Fur with binding buffer
Lane 3: 30 μM of Fur with binding buffer
Lane 4: 75 μM of Fur with binding buffer
Lane 5: 150 μM of Fur with binding buffer
Fig 3.26: EMSA with purified Fur and the region upstream of *rhiX*

Lane 1: Negative control containing no protein, only binding buffer
Lane 2: 15 μM of Fur with binding buffer
Lane 3: 30 μM of Fur with binding buffer
Lane 4: 75 μM of Fur with binding buffer
Lane 5: 150 μM of Fur with binding buffer
Fig 3.27: EMSA with purified Fur and the intergenic region between *fur* and *sitA*

Lane 1: Negative control containing no protein, only binding buffer
Lane 2: 15 μM of Fur with binding buffer
Lane 3: 30 μM of Fur with binding buffer
Lane 4: 75 μM of Fur with binding buffer
Lane 5: 150μM of Fur with binding buffer
The heme transport and the siderophore mediated uptake systems are among the most prominent iron-regulated products of *S. meliloti*. However, the results showed that promoters of the siderophore biosynthesis genes and of the gene encoding its outer membrane receptor along with the heme receptor were not affected by *S. meliloti* Fur in the mobility shift assay.

However, the DNA fragment containing the intergenic region of fur-sitA and with as little as 15 μM of purified Fur was clearly shifted in the gel retardation assay and thus appeared to be bound by *S. meliloti* Fur. Interestingly, the fur-sitA fragment was shifted to two positions. The weakest band, which corresponds to a larger band shift, could be the result of the polymerisation of Fur on the probe. The putative Fur box (63% identity) that is present in the intergenic region could be the binding site of *S. meliloti* Fur.

**3.7.2 EMSA with cell extracts containing overexpressed Fur**

The binding of *S. meliloti* Fur was reassessed in the promoter region of fhuF with the use of *E. coli* XL10 extracts and the results are shown in Fig 3.28. *E. coli* XL10 Gold does not carry a fur mutation and thus the *E. coli* XL10 Gold extracts from cells carrying the empty pQE60 vector were included to control that any band shift observed was not the result of the binding of *E. coli* Fur and not the overexpressed *S. meliloti* Fur.
Fig 3.28: EMSA with *E. coli* extracts from cells carrying either pQE60 or pFUR60 and the region upstream *fhuF*.

Extract used: extract from *E. coli* with pQE60 induced for four hrs with IPTG at a concentration of 0.1 mM

- **Lane 1**: Negative control containing no protein but only binding buffer
- **Lane 2**: 1 µl of *E. coli* extract with binding buffer
- **Lane 3**: 2 µl of *E. coli* extract with binding buffer
- **Lane 4**: 5 µl of *E. coli* extract with binding buffer
- **Lane 5**: 10 µl of *E. coli* extract with binding buffer

Extract used: extract from *E. coli* with pFur60 induced for four hrs with IPTG at a concentration of 0.1 mM

- **Lane 6**: Negative control containing no protein but only binding buffer
- **Lane 7**: 1 µl of *E. coli* extract with binding buffer
- **Lane 8**: 2 µl of *E. coli* extract with binding buffer
- **Lane 9**: 5 µl of *E. coli* extract with binding buffer
- **Lane 10**: 10 µl of *E. coli* extract with binding buffer

The results observed confirmed those obtained with the purified *S. meliloti* Fur. A band shift was detected with *E. coli* XL10 Gold extract transformed with pFUR60. However, no band shift was detected with *E. coli* XL10 Gold, pQE60. This proved that the band shift observed was specific to *S. meliloti* Fur.
The results of the binding of *S. meliloti* Fur to the intergenic region of *fur-sitA* were also confirmed (Fig 3.29).

**Fig 3.29: EMSA with *E. coli* extracts from cells carrying either pQE60 or pFUR60 and the intergenic region between *fur* and *sitA***

Extract used: extract from *E. coli* with pQE60 induced for four hrs with IPTG at a concentration of 0.1 mM

- **Lane 1:** Negative control containing no protein but only binding buffer
- **Lane 2:** 1 μl of *E. coli* extract with binding buffer
- **Lane 3:** 2 μl of *E. coli* extract with binding buffer
- **Lane 4:** 5 μl of *E. coli* extract with binding buffer
- **Lane 5:** 10 μl of *E. coli* extract with binding buffer

Extract used: extract from *E. coli* with pFur60 induced for four hrs with IPTG at a concentration of 0.1 mM

- **Lane 6:** Negative control containing no protein but only binding buffer
- **Lane 7:** 1 μl of *E. coli* extract with binding buffer
- **Lane 8:** 2 μl of *E. coli* extract with binding buffer
- **Lane 9:** 5 μl of *E. coli* extract with binding buffer
- **Lane 10:** 10 μl of *E. coli* extract with binding buffer
3.8 Discussion

This chapter was directed to the investigation of iron regulation of *S. meliloti* through the identification and characterization of a Fur homologue. Earlier work on iron homeostasis suggested that the mechanism of iron regulation in rhizobia might differ from other gram-negative bacteria. This has previously been determined to be the case in two other members of rhizobia, *R. leguminosarum* and *B. japonicum* (Wexler *et al.*, 2003, Nienaber *et al.*, 2001)

The putative fur gene was identified by Blast analysis of the *S. meliloti* genome, which showed a Fur homologue *smc02510* with 41% identity to *E. coli* K12 Fur. The gene encoding this protein is present as a single copy on the chromosome of the bacterium. The *E. coli* Fur protein has been studied in detail and analysis of chimeric proteins, carrying parts of the regulator, indicated that the DNA binding properties are mediated by the N-terminal domain of the protein, whereas the C-terminal domain catalyzes dimerization and binding of the iron cofactor (Stojiljkovic *et al.*, 1995). The fact that both the putative iron binding site HHDH as well as other stretches of amino acids within the C-terminal and N-terminal domain were highly conserved in the *S. meliloti* protein provides evidence for a function similar to its homologue in *E. coli*.

The functional complementation of the fur mutation in *E. coli* confirmed that the *S. meliloti fur* gene is functionally active and interacts with the Fur binding site preceding the *flhF* promoter. The partial complementation of Fur activity in *E. coli* H1681 could be explained by differences in the DNA binding site. For example, another member of the rhizobia, *B. japonicum* provided the first example where a Fur protein binds to DNA in a different way to the usual Fur-'Fur box' DNA binding activity (Friedman *et al.*, 2003). The *B. japonicum* Fur binds to a DNA sequence to which *E. coli* Fur cannot bind. In the results reported here, the interaction was strong enough to allow the study of the influence of iron on regulation and to reveal that the partial suppression of *LacZ* activity mediated by the Fur from *S. meliloti* was...
completely abolished under conditions of iron deprivation. This result provided strong evidence that the *S. meliloti* protein works in a way similar to the Fur proteins of other bacteria and that iron represses *E. coli* *fhuF* suggesting that *S. meliloti* Fur binds to ferrous iron. This result is of particular interest given the roles of the genes regulated by Fur in *S. meliloti* and discussed below. The ability of *S. meliloti* Fur to bind *in vitro* to the promoter region of *E. coli* *fhuF* containing canonical Fur boxes was also examined. The mobility shift assay confirmed the complementation of *E. coli* H1681 and strongly suggested that *S. meliloti* Fur binds to Fur boxes. Similar results were obtained with *B. japonicum* and *R. leguminosarum* Fur homologues, which were also able to respectively complement and partially complement an *E. coli* fur mutant and which can both bind to a canonical ‘Fur box’ (Hamza *et al*., 1999, Wexler *et al*., 2003).

Following this complementation, *S. meliloti* Fur was overexpressed and purified by IMAC. Then, the DNA binding interaction of Fur to the promoter region of iron responsive genes was analysed. Interestingly, Fur did not regulate the biosynthesis of the rhizobactin 1021 siderophore, its permease or its outer membrane receptor. Neither, did it regulate the haem receptor of *S. meliloti*. However, Fur binds to the intergenic region between *fur* and the *sitABCD* operon, which was originally thought to be involved in iron acquisition. However, Platero *et al* (2003) demonstrated that *S. meliloti* mutants in *sitB* and *sitD* were deficient in ferric iron transport and suggested that *sitABCD* are ABC transporters involved in manganese transport and not iron as assumed. The genome of *S. meliloti* reveals the putative *fur* gene next to the *sitABCD* genes and in the opposite orientation. Upstream regions share a perfect palindromic sequence TGCAAATGXXXXX-CATTTGCA. Platero *et al* (2003) suggested a coordinately regulated mechanism for *fur* and *sitABCD* transcription.

It is only recently that transport systems for manganese have been identified. Two main transport mechanisms are dedicated to this task. There are the Nramp proteins (natural resistance-associated macrophage proteins) that are important for controlling bacterial replication and for trafficking metal ions between intracellular compartments. The bacterial Nramp homologues identified to date all appear to
function as Mn\(^{2+}\) and to a lesser degree, Fe\(^{2+}\) uptake transporters under physiological conditions and are named MntH for proton (H\(^{+}\))-dependent Mn transport (Kehres et al., 2000, Makui et al., 2000). The second mechanism is the ABC-type Mn permease system. Characterisation of this now large family of permeases shows that members of the family can transport manganese and in some cases iron and/or zinc. A GenBank search shows this class to be extremely widespread with about twice as many examples as the MntH class. *S. meliloti* Sit ABCD belongs to this class of transporter (Platero et al., 2003, 2004). The mobility shift assays and the complementation suggested that *S. meliloti* Fur can regulate *sitABCD* of *S. meliloti* and *fluF* from *E. coli* and that these genes are repressed respectively by Mn\(^{2+}\) and by Fe\(^{2+}\). Because the chelator 2,2'-dipyridyl binds to Fe\(^{2+}\) and to Mn\(^{2+}\), it cannot be known whether *S. meliloti* Fur binds primarily to iron or to manganese. The partial complementation could be due to the fact that Fur binds more specifically to manganese than to iron.

To date, manganese uptake has been found to be regulated by two main regulators, Fur and MntR. Fur was extensively reviewed in the first chapter, MntR, is the common name of a group for DtxR-like proteins recently identified and including ScaR from *Streptococcus gordonii* (Jakubovics et al., 2000), TroR from *T. pallidum* (Posey et al., 1999) and MntR from *S. aureus* (Horsburgh et al., 2002), *B. subtilis* (Que et al., 2000) and *E. coli* (Patzer et al., 2001). These metalloregressor proteins all function as Mn\(^{2+}\)-dependent transcriptional repressors of genes encoding each type of manganese transporter. When intracellular levels of Mn\(^{2+}\) rise, the DtxR-like proteins bind to an MntR binding motif in the promoter region of the genes and limit transcription. However, an analysis of the genome of *S. meliloti* did not identify any MntR homologues.

Identification of the regulator of some ABC manganese permease operons was easier in cases in which the putative transcriptional regulator is encoded adjacent to or within the operon (Kehres et al., 2003). This is the case for example for SirR in *Staphylococcus epidermidis* that is adjacent to the manganese transport system.
encoded by *sitABC* (Hill *et al.*, 1998) This is also the situation in *S. meliloti* for *sitABCD*, which is adjacent to *fur*.

Other workers have reported that Fur can regulate the transport of manganese in different organisms. First of all, in *E. coli*, the large conventional Fur regulon contains three genes involved in manganese transport *sodA*, *mntH* and *sitABCD* (Fee *et al.*, 1990, Patzer *et al.*, 2001) Also, Fur regulates *sitABCD* and *mntH* in *Salmonella enterica* (Kehres *et al.*, 2000, 2002(a), 2002(b)) It is also interesting to see that in *Yersinia pestis*, Fur is required for repression of *YfeABCD*, encoding an ABC transporter system for both iron and manganese and the expression of this operon can be either repressed by Fe$^{2+}$ or Mn$^{2+}$ (Bearden *et al.*, 1998, 1999) All these transport systems are of the same family of ABC transporters as *sitABCD* in *S. meliloti*. Yet, it is not well understood clearly why transporters involved in manganese acquisition should also be repressed by iron. Also, recently, in *R. leguminosarum*, the Fur-like protein was characterised as being a Mur (Manganese uptake regulator).

The results presented here suggested that Fur in *S. meliloti* is implicated to a greater extent in manganese acquisition regulation and thus could more logically be called a Mur (Manganese uptake regulator). Indeed, so far, it does not regulate any gene involved in ferric iron uptake but solely in manganese acquisition. No other manganese regulator was identified by homology for the maintenance of manganese homeostasis in *S. meliloti*. The findings of this investigation agree with recent publications from two other groups (Platero *et al.*, 2004, Chao *et al.*, 2004) They found through the use of microarrays and reporter gene fusions that the Fur-like protein in *S. meliloti* is a Mur and regulates the *sitABCD* operon encoding the manganese transport system but also the ferrous iron transport system. Yet, through the use of microarrays, Chao *et al.* found that the complete rhizobactin 1021 synthesis operon and the heme receptor encoded by *smc02726* is down regulated in an *S. meliloti fur* mutant. They suggested that the derepression of the *sitABCD* operon led to an increase in intracellular Mn$^{2+}$ and/or Fe$^{2+}$ concentration, which in turn caused the down regulation of the iron utilisation systems. However, the
mobility shift assay performed here contest the ability of *S. meliloti* Fur to bind to the promoter region of those genes and thus their suggestion might still be correct but Fur would not be the transcriptional regulator responsible for the repression of the iron uptake mechanisms.

This is not the first time that a member of the Fur family appears to be having another function than the regulation of iron acquisition. Fur is predominantly an iron-dependent transcriptional regulator of genes involved in iron homeostasis, however its role is not restricted to that and it can for example regulate genes in response to acid pH (Hall et al., 1996). Also, in the characterisation of Fur, it has been demonstrated that Mn\(^{2+}\) can be used to mimic Fe\(^{2+}\) for Fur binding. Mn\(^{2+}\) works as effectively (Schrum et al., 1993) and there is no basis for assuming that iron is necessarily the relevant cofactor for Fur in every case (Kehres et al., 2003). However, there is a risk of misinterpretation of the results as so far in vitro binding experiments are the only results used to identify the co-repressor. Chao et al. (2004) strongly suggested that *S. meliloti* Fur is a Mn\(^{2+}\)-dependent repressor which supports the view that the manganese used in the mobility shift assay is the right Fur co-factor. Also, recent work in *R. leguminosarum* has found that the ability of the Fur-like protein Mur to bind to a canonical Fur box is dependent on iron, not manganese (Diaz-Mireles et al., 2004). However, Mur, which regulates the expression of the *sitABCD* operon in *R. leguminosarum*, is an active repressor in the presence of manganese but has no repressive effect in the presence of iron. A similar mechanism could be the case for *S. meliloti*.

It also emerged over recent years that there exists a family of functionally diverse Fur-like proteins. Genes encoding proteins of this family have been identified including Zur in *E. coli* involved in the maintenance of zinc homeostasis (Gaballa et al., 1998), PerR in *B. subtilis* regulating the manganese response to oxidative stress (Bsat et al., 1998) and Irr which is involved in iron regulation in *B. japonicum*. However, these regulators are distinct from Fur. For example, the *B. japonicum* *irr* gene (Hamza et al., 1998) is related to but is distinct from Fur (29% identical at the amino acid level to Fur of *P. aeruginosa*).
Rhizobial Fur is therefore quite atypical, playing either no role or a much less important role in iron acquisition than homologues in other gram-negative bacteria. In *B. japonicum*, Fur shares the regulation of iron with Irr, and in addition to binding to known Fur boxes binds to additional DNA sequences (Friedman *et al.*, 2003). In *R. leguminosarum*, Fur does not seem to bind the promoter sequences of many Fe-responsive operons that are involved in iron acquisition (Wexler *et al.*, 2003). Finally, *Mesorhizobium loti* does not have any Fur protein homologue.

It is thus clear that the regulation of iron-responsive gene regulation in rhizobia is notably different from other gram-negative bacteria. In the cases studied to date, with the exception of the rhizobia and *Brucella*, Fur is a general regulator of iron acquisition. The analysis of Fur-DNA binding interactions in *S. meliloti* has shown that Fur does not regulate operons usually subject to iron regulation but does regulate at least one manganese acquisition system. A new type of transcriptional regulator *RirA* (*Rhizobial Iron Regulator Activator*) was identified recently in *R. leguminosarum*. *RirA* regulates the expression of iron-responsive operons in this organism. It is interesting to notice that *M. loti*, *S. meliloti* and *B. abortis* each possesses homologues of *RirA*.
Chapter 4:

Identification and characterisation of \textit{rirA} and of \textit{rhrA} in \textit{Sinorhizobium meliloti}
4.1 Introduction

The previous chapter focussed on the global ferric uptake regulator (Fur) homologue in *S. meliloti* given its expected role in regulating iron uptake.

However, the results reported in chapter 3 suggested that Fur is not a regulator of ferric iron uptake but of manganese uptake in this organism. Recently, a new type of iron regulator, called RirA, was identified in *R. leguminosarum*. The mutation of the *rrrA* gene affects the transcription of many genes in response to iron availability. It was thus decided to identify the homologue of this gene in *S. meliloti* and to investigate the role of this regulator concentrating on the role played by *S. meliloti* RirA in regulating the genes involved in the rhizobactin 1021 mediated iron uptake system.

Also, rhizobactin 1021 biosynthesis and transport is known to be regulated by another more specific AraC-like transcriptional regulator, RhrA (Lynch et al., 2001).

In this chapter, a parallel investigation of negative regulation by RirA and positive regulation by RhrA of the siderophore biosynthesis genes and the receptor gene is reported.
4.2 Identification of the *rirA* gene in *S. meliloti* 2011 and Analysis of its encoded product

To identify the *rirA* homologue, the sequence of RirA from *R. leguminosarum* was used to perform a BLASTP, which compares an amino acid query sequence against a protein sequence database of the *S. meliloti* 2011 genome.

Four proteins were obtained from the blast: SMc02238, Smc02267 and Smb20994 with respectively 34 %, 26 % and 29 % similarity and, with 84 % similarity, SMc00785, the closest homologue of RirA in *S. meliloti* 2011 (Table 4.1).

Table 4.1: BlastP results with *R. leguminosarum* RirA as the query sequence

<table>
<thead>
<tr>
<th>BlastP results: Hit Description</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rirA</em></td>
<td>SMc00785_AA (154 aa)</td>
</tr>
<tr>
<td>CONSERVED HYPOTHETICAL PROTEIN</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Begin position</td>
<td>End position</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>1</td>
<td>432</td>
</tr>
<tr>
<td>Blast score</td>
<td>617</td>
</tr>
<tr>
<td>Identity</td>
<td>84%</td>
</tr>
</tbody>
</table>

The hit concerns 89% of the query sequence and 92% of SMc00785_AA.

In the *S. meliloti* annotated genome (Galibert *et al*., 2001), SMc00785 was originally termed *aau3*, as it was thought to specify a protein involved in acetoacetate utilization (Charles *et al*., 1997). However, *aau3* is, in fact, elsewhere in the genome and SMc00785 had so far no known function (Todd *et al*., 2002).

An interesting feature of this region of the genome is that the gene immediately downstream of *smc00785*, *dppA1*, encodes a homologue to an heme-transporter involved in iron uptake (Table 4.2). *S. meliloti* *dppA1* is homologue to *R. leguminosarum* *dppA1*.
leguminosarum dppA, which is part of the dppABCDF operon. Those genes are required to transport dipeptides in bacteria. dpp mutants, in R. leguminosarum, were severely affected in the import of delta-aminolevulinic acid (ALA), a heme precursor (Carter et al., 2002).

Table: 4.2: Iron ABC transporter with homology to DppA1 of S. meliloti

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Accession</th>
<th>Homology</th>
</tr>
</thead>
</table>
| DppA1 (Heme-binding protein) | Rhizobium leguminosarum bv. viciae | CAC35511 | 75 % identity  
85 % similarity |
| DppA1 (Heme-binding lipoprotein) | Haemophilus influenzae | NP_439013 | 50 % identity  
69 % similarity |

The deduced protein product (SMc00785) of rirA has very close homologues (Fig 4.1) in R. leguminosarum (84 % identity) and Agrobacterium tumefaciens (85 % identity). In A. tumefaciens, the gene was also erroneously termed aau3, and, as in S. meliloti, is adjacent to a gene that is likely to specify an inorganic Fe\(^{3+}\) transporter. Other homologues of RirA also occur in Mesorhizobium loti (65% identity) and in Brucella suis and Brucella melitensis, which are not members of the rhizobia, but which also have RirA-like proteins (66% identity in both cases) of no known function. Todd et al. (2002) noted that the corresponding gene in Brucella is separated by two ORFs from a homologue of a bacterioferritin gene. These are the only homologues of RirA found in the genomes sequenced to date.

By performing a BLASTP program on S. meliloti RirA using the NCBI database of protein sequences (Altschul et al., 1997), it was also concluded that RirA is part of the family of proteins called RrF2. These are small proteins of 12 to 18 KDa, which seem to contain a signal sequence, and may represent a family of probable transcriptional regulators. Most RrF2 proteins possess 3 cysteines in their C-terminal (Fig 4.1). The cysteine could be the site to which the ferric iron, being the cofactor, binds to the transcriptional regulator, which would allow RirA to bind to the promoter region of the gene it regulates.
Fig 4.1: Comparison of *S. meliloti* RirA (SMc00785) to other RirA homologues.

The *S. meliloti* RirA protein is aligned with very close homologues from *R. leguminosarum*, *A. tumefaciens*, *M. loti*, and *B. melitensis*. The orange circle shows the three C-terminal cysteines characteristic of RrF2 proteins.
4.3 Mutation of Smc00785, the rirA homologue in S. meliloti 2011

After the identification of the S. meliloti rirA homologue Smc00785, which was subsequently called S. meliloti rirA, a major objective was to mutate the gene with the use of a kanamycin cassette and to investigate the phenotype of the mutant.

The method used involved cloning the cassette into the S. meliloti rirA homologue gene in E. coli. The mutated gene was then mobilised to S. meliloti in a suicide vector. Selection was made for a single recombination that left the vector integrated in the S. meliloti genome. A second recombination event was then selected, expelling the vector and leaving the cassette inserted in the genome.

This method is facilitated by the use of a pJQ200sk (Quandt and Hynes, 1993), a suicide vector permitting mobilisation and gene replacement in a wide range of Gram negative bacteria. This vector was used to insert fragments via recombination into the chromosome of S. meliloti 2011. This vector possesses a gentamicin resistance marker, a multiple cloning site from pBluescript KS+ (see Fig 4.2) and a mob (oriT) site, which facilitates the mobilization into S. meliloti. Finally, the sacB gene is lethal in a wide range of Gram negative bacteria when grown on media containing 5% sucrose, and thus permits a positive selection for the loss of the vector, which occurs during the second recombination event shown in the mutagenesis scheme in Fig 4.3.
Fig 4.2: Map and Multiple cloning site (MCS) of pJQ200sk (Quandt and Hynes, 1993), gm; gentamicin resistance, tra; transfer.
Fig 4.3: Recombination event scheme.

A', B', C' denote the copies of A, B, C cloned into the pJQ200sk vector. The kanamycin resistance cassette is inserted in B' (Δ). The sites of recombination events are indicated (X).
The targeted mutation was made using a kanamycin resistance cassette from pUC4K (Vieira and Messing, 1982). This cassette is flanked by sites for five commonly used restriction enzymes *EcoRI, BamHI, Sail, HincII* and *PstI* (See Fig. 4.4). The presence of these sites enables the cassette to be cloned into similar restriction sites, or sites that are cleaved by restriction enzymes that produce compatible ends to the enzymes bounding the cassette. Because transcriptional regulators are generally small genes that usually only have suitable restriction sites for uncommon restriction enzymes, the kanamycin cassette had to be amplified by PCR from pUC4K incorporating new restriction sites in the primers.

Fig 4.4: Map of pUC4K (Vieira and Messing, 1982) with kanamycin resistance cassette.
A restriction analysis was carried out of the *rirA* region of *S. meliloti* (see Fig 4.5) to find a suitable restriction site to insert the kanamycin resistance cassette (see Fig 4.6). The enzymes that only cut the *smc00785* gene once are shown in Fig 4.6. The sequence analysis of *rirA* revealed the presence of a unique *Ncol* site within the gene into which the kanamycin resistance cassette could be inserted.
Fig 4.6 Enzymes that only cut the *rirA* gene once

Two primers *rirA*-F and *rirA*-R were designed to amplify an approximatively 20 Kb region of the *S. meliloti* 2011 genome encoding *rirA*, with the *NcoI* site centrally located. The forward primer *rirA*-F was designed so as to incorporate a unique *XhoI* site into the PCR product. The reverse primer *rirA*-R was designed to incorporate a unique *Spel* site into the PCR product. The unique *Spel* and *XhoI* sites in the PCR product were added to allow for the subsequent directional cloning of the 20 Kb fragment into pJQ200sk.

Total genomic DNA was prepared from *S. meliloti* 2011 and used as the template DNA in the PCR reaction. Following optimisation of the PCR reaction, a specific 20 Kb PCR product was obtained and cloned into the pCR2.1 vector. The 20 Kb fragment was restricted from pCR2.1 as an *XhoI/Spel* fragment and cloned directionally into pJQ200sk. The kanamycin cassette was amplified as an *NcoI* fragment and inserted into the unique *NcoI* site of the pJQ200sk *rirA* plasmid.

The diagram in Fig 4.7 summarises the strategy used to construct the final clone, called pRirA200K.

**PCR Conditions**

- PCR reaction for the amplification of *rirA* with its flanking regions from a genomic prep of *S. meliloti* 2011

The primers used to amplify *rirA* and its flanking sequences on each side (*XhoI/Spel* fragment) were:

**rirA-F** CTCGAG TCACCGAGGACTTCTCTG

*XhoI*

**rirA-R** ACTAGTGAA GTCCGCCTGTAACGCTATGCG

*Spel*
The PCR program of the reaction is summarised in Table 4.3.

Table 4.3: PCR Reaction Conditions for the amplification of the *S. meliloti rirA* and its flanking regions.

<table>
<thead>
<tr>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealing Temperature 68°C</td>
</tr>
<tr>
<td>Annealing Time 1 min</td>
</tr>
<tr>
<td>Extension Time 72°C for 3 min</td>
</tr>
</tbody>
</table>

*PCR reaction for the amplification of the kanamycin cassette from pUC4K:*

The primers used to amplify the kanamycin resistance cassette from pUC4K as an *NcoI* fragment were:

**Kan*NcoI*-F: CCATGG GAC GTT GTA AAA CGA CGG CCA GTG**

*NcoI*

**Kan*NcoI*-R: CCATGG GGA AAC AGC TAT GAC CAT GAT TAC G**

*NcoI*

The PCR program of the reaction is summarised in Table 4.4.
Table 4.4: PCR Reaction Conditions for the amplification of the kanamycin resistance cassette from pUC4K

<table>
<thead>
<tr>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealing Temperature 64°C</td>
</tr>
<tr>
<td>Annealing Time 1 min</td>
</tr>
<tr>
<td>Extension Time 72°C for 1.5 min</td>
</tr>
</tbody>
</table>

Total genomic DNA from *S. meliloti* 2011

**Fig. 4.7: Strategy for the mutation of *S. meliloti* rirA (smc00785) gene**
The plasmid was introduced into *Sinorhizobium meliloti* 2011 by triparental mating and transconjugants were selected on TY containing streptomycin and gentamicin. Second recombinants were selected by growing a clone that had undergone a single first recombination without antibiotic selection in TY broth until early stationary phase and then plating on TY agar containing 5% sucrose and kanamycin. Individual colonies were then screened for kanamycin resistance and gentamicin sensitivity.

**Confirmation of the *rirA* mutation**

The mutation of *rirA* was confirmed using PCR by a comparison of the PCR products obtained from *Sinorhizobium meliloti* 2011 and from the mutant strain *Sinorhizobium meliloti* 2011*rirA*2 following the amplification of the *rirA* region. The region would be around 1.4 Kb larger due to the insertion of the kanamycin resistance cassette (Fig 4.8). The mutant was named *Sinorhizobium meliloti* 2011*rirA*2.
Fig 4.8: PCR to confirm mutation of the chromosomal \textit{rirA} gene

Lane 1: 1 Kb ladder
Lane 2: Chromosomal prep from \textit{S. meliloti} 2011
Lane 3: \textit{rirA} PCR using \textit{rirA}-F and \textit{rirA}-R on \textit{S. meliloti} 2011
Lane 4: Chromosomal prep from \textit{S. meliloti} 2011\textit{rirA}2
Lane 5: \textit{rirA} PCR using \textit{rirA}-F and \textit{rirA}-R on \textit{S. meliloti} 2011\textit{rirA}2
The mutation was also confirmed by Southern hybridisation. The genomic sequence in the region encoding \textit{rirA} was examined to identify restriction sites that were deemed suitable for the confirmation of the potential mutant by Southern hybridisation analysis. The kanamycin resistance cassette was inserted into an \textit{NcoI} site encoded within a 5.9 Kb \textit{XhoI}-\textit{XhoI} fragment (Fig 4.9) as an \textit{NcoI} fragment. Digestion of the mutant genomic DNA would generate one fragment with an \textit{XhoI} digestion (Fig 4.10). The plasmid pRirA200K was labeled and used as a probe.

\textbf{Fig. 4.9:} The region encoding \textit{rirA} in \textit{S. meliloti} showing the \textit{XhoI} and \textit{NcoI} restriction sites and the fragment sizes that would hybridise with the pRirA200K probe. The labeled probe is indicated in red, while the kanamycin resistance cassette is highlighted in green. Regions of homology between the labeled probe and the digested fragments are indicted.
Genomic DNA was prepared from *S. meliloti* 2011 and the potential mutant and then restricted with *XhoI*, transferred to nitrocellulose and probes with labeled plasmid as described in chapter 2. Examination of the hybridization result indicated that the kanamycin cassette had integrated correctly into the chromosome (Fig 4.11).
Fig 4. 11: Southern hybridisation analysis of the *S. meliloti* 2011 and *S. meliloti* 2011*rirA2* confirming the correct insertion of the kanamycin resistance cassette.

Lane 1: 1 Kb Ladder
Lane 2: *S. meliloti* 2011 *Xhol*
Lane 3: none
Lane 4: *S. meliloti* 2011*rirA2 Xhol*
4.4 Phenotypic Analysis Of the S. *meliloti rirA* mutant

One role of the general regulator Fur in, for example *E. coli*, is to downregulate the expression of the siderophore. However, as shown in the previous chapter, this is not the case for the Fur homologue in *S. meliloti*. It was hypothesised that RirA could fulfil this function. Having constructed the *S. meliloti rirA* mutant, it was possible to determine the function of the gene, with regard to the regulation of the iron response, by comparison with the wild type. This was undertaken by investigating primarily the production and utilisation of rhizobactin 1021. Initially, the production of rhizobactin 1021 was examined by the plate bioassay.
4.4.1 The siderophore plate bioassay

The siderophore plate bioassay is based on the promotion of bacterial growth by siderophores in a medium where traces of iron are removed by an iron chelator. The bioassay was carried out with TY medium in which a chelator, 2,2'-dipyridyl, was added to remove any trace of free iron and wells were made in the medium to place the different control and siderophore preparations (Fig. 4.12).

Fig. 4.12: Siderophore Plate Assay
Siderophore preparations were made from the following sources

- *S. meliloti* 2011 grown under iron replete conditions in which no siderophore is expected to be produced
- *S. meliloti* 2011 grown under iron deplete conditions in which under iron stress, the bacteria will produce the siderophore
- *S. meliloti* 2011*rirA2* grown under iron replete conditions, in which case, it will be assessed whether *RirA* regulates the rhizobactin 1021 biosynthesis operon

Thus, these three siderophore preparations, plus a solution of ferric chloride as a positive control were placed in different wells made in the TY media supplemented with 2,2'-dipyridyl. The medium was seeded with 200 μl of a late logarithmic culture of 2011*rhbA62*, a siderophore biosynthesis mutant.
4.4.2 Phenotypic analysis of *S. meliloti* 201IrirA2 by the siderophore plate bioassay

Fig 4.13: Siderophore plate bioassay

A: FeCl₃  
B: Iron replete conditions, *S. meliloti* 2011 siderophore preparation  
C: Iron deplete conditions, *S. meliloti* 2011 siderophore preparation  
D: Iron replete conditions, *S. meliloti* 2011IrirA2 siderophore preparation
As expected in the negative control, the siderophore preparation from iron replete grown *S. meliloti* 2011, no halo of *S. meliloti* 2011*rhbA62* growth was observed as the strain could not grow in the presence of the iron chelator. This was in contrast to the preparation of the same strain grown under iron deplete conditions. In the positive control, ferric chloride, the siderophore biosynthesis mutant could utilise the abundant inorganic iron producing a halo of growth around the control well. With the siderophore preparation of interest from *S. meliloti* 2011*rirA2* grown under iron replete conditions, a halo of growth surrounded the well implying that *S. meliloti* 2011*rirA2* is able to produce the rhizobactin 1021 siderophore despite the presence of iron. It can be concluded that RirA from *S. meliloti* is involved in the regulation by iron of the production of rhizobactin 1021, which was constitutively produced in the *rirA* mutant even under iron replete conditions. The next step was to consider if RirA was acting at the transcriptional level binding directly under iron replete conditions to the promoter region of the rhizobactin 1021 biosynthesis gene cluster.
4.5 In vivo genetic manipulations to analyse the iron responsive rhizobactin 1021 biosynthesis operon.

4.5.1 Principle and design of the probes

The approach taken to investigate the binding of RirA in vivo was to construct promoter probes with the promoter region upstream of \( rhtX \) (Fig 3 1) fused to a reporter gene. Those promoter probes would also allow an investigation of the role of RhrA, the AraC-like activator, under iron deplete conditions regarding the activation of the siderophore genes.

Thus, in order to examine the binding of the two transcriptional regulators RirA and RhrA, a plasmid-based promoter probe vector pOT-1 was used (Fig 4 14). Its reporter gene is \( gfpuv \), which has a 18-fold increase in fluorescence relative to the wild-type \( gfp \) but retains the latter’s excitation and emission maxima of 495 and 510 nm respectively (Crameri et al., 1995). This vector was chosen as it is a small broad host range vector with a medium copy number, which thus limits any possible titration effect, which could occur especially with RhrA that may be present in low abundance in the cell. Also, the vector is mobilisable and is stably maintained in Gram-negative bacteria. Finally, its gentamicin resistance makes it suitable for studies in \( S \) meliloti.
Fig. 4.14: Map of pOT1

A. pOT-1 has the gfpuv reporter gene flanked by the omega and rrnB transcriptional terminators. An artificial ribosomal binding site (RBS) was introduced to the 5' primer to gfpuv.

B. The polylinker of pOT-1.
To investigate the binding of RirA and RhrA, the upstream region of \textit{rhtXrhhABCDEF} was fused to the reporter gene. Usually, as explained in chapter one, AraC-transcriptional regulators bind as dimers to the promoter regions on 17-bp repeats separated by 4-bp. Two repeats GTTCGC with an inter region of 15 bp are found upstream of the operon and look like good candidates for the binding of RhrA. In order to confirm those predictions different mutations of the sequence were made (Fig 4.15).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{promoter_design.png}
\caption{Design of the promoter probes
A: The \textit{rhtX} promoter region. The two repeats upstream of \textit{rhtX} are framed. B: Plasmid constructs in which the R1 and R2 repeats have been altered.}
\end{figure}
Five clones were constructed with different variations of the promoter region of the operon cloned into pOT1

- **pWT** The wild type promoter region sequence with the two binding sites present was cloned into the multiple cloning site of the vector as a *HindIII-PstI* fragment

- **pM1** The upstream region with the sequence excluding the distal repeat was cloned into the multiple cloning site of the vector as a *HindIII-PstI* fragment

- **pEN2** The proximal repeat was removed but the distal repeat conserved

- **pEN3** The two repeats and their intergenic region were removed from the promoter region

- **pEN4** An extra 6 bases was added by inserting a *BglII* site between the two repeats

Construction of pWT and pM1 was straightforward and involved a single PCR. For the construction of pEN2, pEN3, pEN4, it was necessary to undertake two PCR steps using pWT as template DNA as described in Fig 4 for pEN2 for example, and then to undertake a three fragment ligation to join the two PCR products together and ligate them to the vector. To do so, the region that had to be conserved was amplified from pWT as two separate fragments - an *Ncol-BglII* fragment (PCR 1) and a *BglII-EcoRI* fragment (PCR 2) *BglII*, which had no site in the region of interest, was used to replace the deleted region. Once the PCR was performed and then the product cleaned, it was restricted with the appropriate restriction enzymes and ligated with the *Ncol-EcoRI* restricted pOT1 vector and the transformants were then selected on medium containing gentamicin.
Fig 4.16: The PCRs performed on pWT for the cloning of pEN2

The following primers were designed for the amplification of the \textit{rhtX} promoter region and for the mutagenesis of that region:

**Primers for the construction of pWT:** Insertion of the upstream sequence of \textit{rhtX} in pOT1 as \textit{HindIII/PstI} fragment

\begin{verbatim}
HindIII
AAGCTT CCCT GGAACGGTCG TATCGGCTCT CTCGAAAATG CTTGCGGAC
TGCTTTAATG ACGTTCACC TGTCGCCCGC ACGTCCATT
AAAGATGACC GCAACACTCA TGGTATCGT CAGACATGG TGCC TCGAG
\end{verbatim}
The PCR products were amplified, cleaned, restricted with *HindIII*/*PstI* and inserted in pOT1, which had been restricted with *HindIII*/*PstI*.

**Primers for the construction of pM1: Insertion of the upstream sequence of rhtX from downstream of the distal repeat R1 in pOT1 as *HindIII*/*PstI* fragment**

![HindIII Primer](image)

**F-rhrAMl:**

TTT AAGCTT TAC TGT CTT AAT GAG GTT CGC TCA C

**R-rhrAWT**

AAAA CTGCAG GGC AAC ATT GTC TGA CGA TAA ACA TG

The PCR products were amplified, cleaned, restricted with *HindIII*/*PstI* and inserted in pOT1, which had been restricted with *HindIII*/*PstI*. 

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Specific primers were designed for the amplification of the specific fragments of p-WT to mutate the promoter region of \textit{rhtX} cloned into p-WT. The following sequences and primers summarise the different strategies employed:

\textbf{Primers for the construction of p-EN2: Promoter probe where the proximal repeat of the \textit{rhtX} promoter region of p-WT is removed:}

\begin{verbatim}
HindIII  AAGCTTCCCT GGAGGCGTCC TATCGCCTCT CTCGAAAATG CTTTCGCTAC
BgII  TGTCTTAATG AG     TC ACATCCAAGC CGTTACCGC ACGTCCATTT
AAAGATGACG GCAACACTCA TGTTTATCGT CAGACAATGT GTCACGTCAC

\textbullet{} PCR 1:
F-NcoIpOT1:
CAGT CCACTGG GCA AAT GGG ATT GGC
R-BgIIIR2:
GA AGATCT CTC ATT AAG ACA GTA GCG AAC GC

\textbullet{} PCR 2:
F-BgIIIR2:
GA AGATCT TCA CAT CCA AGC CGT TCA CCG C
R-EcoRIpOT-1:
CG GAATTCC ATT ATT TGT AGA GCT CAT CC
\end{verbatim}
Primers for the construction of p-EN3: promoter probe where both repeats the region from the proximal to the distal repeats of the *rhtX* promoter region of p-WT are removed:

**HindIII**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-NcoIpOT-1</td>
<td>CAGTCCTCATGGGCAATGGATTGC</td>
<td>NcoI</td>
</tr>
<tr>
<td>R-BglIIpOT-1</td>
<td>CGAAGATCTGCAAATTTGAGGGCGTCAAGATCTTCAAGGCAACACCTCAAGATCT</td>
<td>BglII</td>
</tr>
</tbody>
</table>

♦ PCR 1:

F-NcoIpOT-1:

CAGTCCATGGGCAATGGATTGC

R-BglIIpOT-1:

GAAGATCTGCAAATTTGAGGGCGTCAAGATCT

♦ PCR 2:

F-BglIIpOT-2:

GAAGATCTGCAAATTTGAGGGCGTCAAGATCT

R-EcoRIpOT-1:

CGGAATTCATTATTGTAGACTCAAGATCT
Primers for the construction of P-EN4: Promoter probe where an additional six bases were added upstream of the proximal repeat of the \textit{rhtX} promoter region of p-WT:

\textit{HindIII}  
\textbf{AAGCTT|CCCT GGAGCGTCC TATCGCCTCT CTCAAAAATG CTGTTCCGTCAC}

\textit{BglII}  
TGTCTTAATG AG\textbf{AGATCT} GTTCGCCGC ACATCCAAGC CGTTCAAGCC ACGTCCATTT

\textbf{PCR 1:}  
F-NcoIpOT-1:  
\textbf{CAGT CCA}GG GCA AAT GGG ATT GGC

R-BglIIIR2:  
GA AGATCT TCA CAT CCA AGC CGT TCA CCG C

\textbf{PCR 2:}  
F-BglII+6:  
\textbf{AGATC}T GTT CGC TCA CAT CCA AGC CGT TC

R-EcoRIpOT-1:  
\textbf{CG GAA}ATC ATT ATT TGT AGA GCT CAT CC
The PCR reaction program was the same for all the fragments amplified and is summarised in the following Table 4.4:

Table 4.4: PCR Reaction Conditions for the amplification of the *S. meliloti* upstream region of *rhtXrhhABCDEF*.

<table>
<thead>
<tr>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealing Temperature</td>
</tr>
<tr>
<td>Annealing Time</td>
</tr>
<tr>
<td>Extension Time</td>
</tr>
</tbody>
</table>

The constructed plasmids were then mobilised into three different strains:
- *S. meliloti* 2011
- *S. meliloti* 2011*rirA2*, a *rirA* mutant
- *S. meliloti* 2011*rhrA26*, a *rhrA* mutant

The analysis of the quantitative level of GFP activity was based on the method used by Tang *et al.* (1999). TY in this case was used as the blank and the *S. meliloti* strain carrying the empty vector pOT1 as the control. The relative fluorescence intensity ($I_R$) was calculated based on the following formula:

$$I_R = \frac{I_{abs}}{OD_{600}} - \frac{I_C}{OD_C}$$

Where $I_C$ is the $I_{abs}$ of *S. meliloti* carrying the empty vector; $OD_C$ is the $OD_{600}$ of *S. meliloti* carrying the empty vector.
4.5.2 Expression of the GFP reporter fused to wild type and mutated rhtXrhhABCDEF promoter sequences and measured in the wild type, rirA and rhrA26 backgrounds

First, the binding activity of RirA to the promoter region was investigated under iron replete conditions with 2011 [pOT1] and 2011rirA2 [pOT1] were used as negative controls. A comparison of the GFP level was made between S. meliloti 2011 [pWT] and S. meliloti 2011rirA2 [pWT]. The cultures were grown to late exponential phase and their GFP activity measured (Fig 4.17) as explained in chapter 2.

The results indicate an increased level of the reporter gene expression in the *rirA2* mutant. The levels of fluorescence, normalised with the strain containing the empty vector, were calculated to have a 9 fold-increase in *S. meliloti 2011 rirA2* [pWT] compared to *S. meliloti 2011* [pWT]. The results were also confirmed by examining the culture under a microscope. A drop of each culture was placed on a slide and the culture flamed in order to fix the moving bacteria.

The slides were then viewed under bright light and UV light (Fig 4.18, Fig 4.19).

Fig 4.18: Culture of *S. meliloti 2011* [pOT1] (A and B) and *S. meliloti 2011* [pWT] (C and D) under bright light to confirm the presence of the bacteria (A and C) and UV light for green fluorescent protein (B and D). Magnification 1000 X.
These findings demonstrate that under iron replete conditions, the $gfp$ gene is only expressed in the \textit{rirA2} mutant \textit{S. meliloti} 2011\textit{rirA2} \{pWT\}. The plasmid pWT contains the iron-responsive \textit{rhtX} promoter region, fused to a $gfp$ reporter gene in the wide-host-range promoter-probe plasmid pOT1. This suggests that the \textit{S. meliloti rirA} mutant is defective in the iron dependent repression of the expression of the operon \textit{rhtXrhhABCDEF} and thus the production of the siderophore rhizobactin 1021 is under the control of RirA. Therefore, under iron replete conditions, this regulator represses the expression of the biosynthesis genes at the transcriptional level, possibly binding directly to the promoter region of the gene it down regulates.
Following the outcome of the previous experiment, the promoter probe plasmids with deletions in the promoter region were used to attempt to isolate a putative binding site for RirA. Each of the promoter probe plasmids was conjugated into \textit{S. meliloti} 2011 were grown under iron replete conditions until late exponential phase and then examined for GFP activity.

![GFP activity chart](image)

**Fig 4.20: GFP activity of the different promoter probes under iron replete conditions in \textit{S. meliloti} 2011.**
(because it has the higher level relative level of fluorescence under iron replete conditions: 100% was given to 2011rirA2 pWT)

GFP activity was only detected in \textit{S. meliloti} 2011 [pM1]. The levels of fluorescence, normalised with the strain containing the empty vector, were calculated to have a 14 fold-increase in \textit{S. meliloti} 2011 [pM1] compared to \textit{S. meliloti} 2011 [pWT]. Since pM1 is deleted for 35-bp of the sequence (See Fig 4.15), this indicates that some or all of the first 35-bp of the sequence cloned in pWT is involved in the binding of RirA (See Fig 4.20).
RhrA is the activator of the \textit{rhtXrhhABCDEF} promoter under iron deplete conditions. The activation by RhrA of the mutated promoter was therefore investigated. Each of the plasmids carrying mutated promoters was conjugated into \textit{S. meliloti 2011}, grown under iron deplete conditions until late exponential phase and examined for GFP activity. The results are shown in Fig 4.21.

![GFP activity graph](image)

\textbf{Fig 4.21:} GFP activity of the mutated promoter fusions under iron deplete conditions in \textit{S. meliloti 2011}. The negative control was \textit{S. meliloti 2011 2011rhr426} for which none of the promoter probes gave any GFP activity.

These findings demonstrate that under iron deplete conditions, the \textit{gfp} gene is only highly expressed in \textit{S. meliloti 2011 [pWT]} and \textit{S. meliloti 2011 [pM1]}. The plasmid pWT contains the iron responsive \textit{rhtXrhhABCDEF} promoter region, fused to a \textit{gfp} reporter gene in the wide-host-range promoter-probe plasmid pOT1 and the plasmid pM1 contains the iron responsive \textit{rhtX} promoter region, fused to a \textit{gfp} reporter gene but with 35-bp of the upstream region removed resulting in the removal of the distal repeat sequence. This suggests that the distal repeat is not necessary for RhrA activation of the promoter, although the level of activation was shifted lower in the case of pM1, suggesting that its presence may affect the efficiency of activation. In the cases of pEN2, pEN3 and pEN4, no activation was detected. pEN2 and pEN3 both lack the proximal repeat, while pEN4 has an insertion that disrupts the base
sequence beside the proximal repeat. The results imply that the proximal repeat is critical in the activation by RhrA of the reporter under iron deplete conditions.
4.6 Analysis of the level of transcript of iron responsive genes using Real time RT-PCR

Real time RT-PCR can be used as an alternative to the RNase Protection assay or Northern hybridisation to analyse gene expression and was utilized to investigate RhrA and RirA regulation at the RNA level.

Real-time RT-PCR is a technique that has been widely used to estimate the levels of expression of genes, especially in eukaryotes. An optimised real-time RT-PCR assay is almost as reproducible as a real-time PCR assay. However, the critical issues defining the reliability of the obtained data are the choice of the housekeeping gene and RNA sample preparation. An ideal housekeeping gene should have the same level of expression under different conditions of growth. For eukaryotes, stably expressed housekeeping genes such as beta-actin can be used as standards to perform a quantification of gene expression (Bustin et al., 2000). Unfortunately, for bacteria no such stably expressed gene is really known. To date, the most widely and housekeeping gene is 16s rRNA (Neretin et al., 2003). Accurate quantification of RNA species is still difficult with prokaryotes because of the absence of a reliable standardised housekeeping genes.

For some applications, such as the influence of iron deplete and replete conditions, a relative quantification is sufficient (Klein, 2002). In those cases, the development of accurate RNA standards can be avoided by using a comparative quantification method. The method is based on the ratio between the amount of target molecule and a reference molecule. This normalised value can then be used to compare differential gene expression in different samples.

The real-time RT-PCR used in this work is based on a non-specific detection system. The standard method for non-specific detection is a double stranded DNA intercalating dye that fluoresces once bound to the DNA. The most commonly used
dye is SYBR Green™ I. This dye binds to all double stranded DNA molecules emitting a fluorescent signal on binding.

Specific primers (Table 4.5) were designed to amplify the *S. meliloti* genes shown in Table 4.5 and to study their levels of expression under different conditions of growth and in different *S. meliloti* strains. The size of the DNA sequences amplified by the primers was between 150-200 bp, which is the optimal size for real-time RT-PCR.

**Table 4.5: Real time RT-PCR primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
</table>
| *rhrA* | RhbA-F: ATG CCG GCC GAT TTA GCC  
RhbA-R: TCG CGT CTT TCC TGT CCG |
| *rhtA* | RhtA-F: CTATGGAAATTGGCAAACCTACTC  
RhtA-R: CGATGATCTCAACGGCAAGC |
| *rhrA* | RhrA-F: TGC CAG CGA CAG GGA AAC G  
RhrA-R: ATG GAG ACA ATC CGA CCG |
| *dppAl* | dppA1-F: CAC TAC TCT TCT TGG AGC G  
dppA1-R: ACG GCT GTA AAC GGT ATG CG |
| *rirA* | rirA-F: GCG TCT GAC GAA GCA AAC C  
rirA-R: GCG TCT GAC GAA GCA AAC |
| *Smc02726* | Smc02726-F: TGCTCAAACGGCATCATCGCCTGGC  
Smc02726-R: CGCGACGATCTTCTTTCAGCACGGTC |
| *16S rRNA* | 16S rRNA-F: ACT TGA GAG TTT GAT CCT GGC  
16S rRNA-F: TCT TTC CCC CGA AGG GCT C |
| *npt* | npt-F: CGC AGG TTC TCCGGC CGC  
npt-R: CTG CGC AAG GAA CGC CGC |

The primers’ specificities and efficiencies were checked by PCR amplification using *S. meliloti* genomic DNA as template.

Prior to the RT-reaction, a DNase treatment was performed on the RNA preparations to eliminate any contaminating DNA from the RNA preparations. Real time RT reactions were set up as described in chapter 2. The *npt* gene from the transposon Tn5 and 16S rRNA gene were used as housekeeping genes because they are considered as stable references (Lynch et al., 2001; Neretin et al., 2003). The
amplification program consisted of 1 cycle of 95°C for 15 minutes followed by 50 cycles of 95°C with 20-s hold, a specified annealing temperature of 56°C with 30-s hold and 72°C with 30-s hold. The generation of specific PCR products was confirmed by melting curve analysis and gel electrophoresis. Each quantitative real-time RT-PCR experiment was performed in triplicate.

Fig 4.22 shows the melting curve analysis of the rirA gene of S. meliloti exposed under iron replete conditions as an example. The melting curve shows a single peak with a melting temperature above 80°C. Peaks with a melting temperature below the value are usually primer-dimer extensions.

The results of the melting curve were confirmed by gel electrophoresis with the expected size of the product, which is 150 bp (Fig 4.23).
Fig 4.23: 2% agarose DNA gel electrophoresis of the PCR product using primers for *rirA*

Lane 1: Ladder

Lane 2: Real-time RT-PCR product of *rirA*
In some cases, the melting curve showed a peak at a lower temperature than that of the specific PCR product, often below 70°C. This was probably the result of the formation of primer-dimers. In most cases, the problems were abolished by diluting the concentration of primers from a concentration of 4 µM to a concentration of 0.4 µM. A primer concentration that is too high can increase the yield of non-specific products. In other cases, however, the dilution of the primers or the optimisation of the annealing temperature was not enough and new primers had to be designed for some genes. The magnesium chloride concentration could also be a factor affecting the formation of primer-dimers or unspecific products, however as it is part of the SYBR Green Master mix, it would have been difficult to vary the concentration and optimise it.

For the analysis of the level of transcripts of iron-responsive genes, relative quantification, which is the most widely used technique in real-time RT PCR, was used. For this method of quantification, an endogenous control was amplified from the sample as well as the gene(s) of interest. By using an endogenous control as an active reference, quantification of an mRNA target can be normalised for removing errors caused by slight variation in PCR efficiencies between samples and different amounts of template. The endogenous control was compared to Ct (Ct first cycle at which the fluorescent signal obtained during real-time RT-PCR is significantly higher than the background signal) values and the following equations used:

\[ \Delta Ct = \text{endogenous } Ct - \text{Gene of interest } Ct \]

\[ \Delta \Delta Ct = \Delta Ct \text{ of sample} - \Delta Ct \text{ of calibrator} \]

Amount of target normalised to a control and relative to a calibrator =

\[ 2^{(\Delta \Delta Ct)} \]
4.6.1 Regulation of rirA as detected by real time RT-PCR.

Given the possible role of RirA in regulating iron uptake, the effects of iron on the expression of \textit{rirA} itself were determined. To do this, a real-time RT-PCR was performed comparing the level of mRNA of \textit{rirA} in \textit{S. meliloti} under iron deplete and replete conditions. In this experiment, the housekeeping gene chosen was \textit{npt}.

![Fig 4.24: In vivo analysis of the iron regulation of \textit{rirA} by Real-Time PCR](image)

(Fe+): Iron replete condition (Fe-): Iron deplete condition

The real-time RT-PCR (Fig 4.24) clearly showed that the expression of \textit{rirA} is iron regulated. The expression of the gene is undoubtedly down regulated under iron deplete conditions compared to iron replete, with a level of transcripts near 0.
4.6.2 Iron regulation of \textit{rhbA} and \textit{rhtA} as detected by real time RT-PCR.

The effect of iron was also determined on the expression of \textit{S. meliloti} genes that are potentially regulated by RirA. To do this, a real-time RT-PCR was performed comparing the level of mRNA of \textit{rhbA} and \textit{rhtA}, which are genes involved in the siderophore mediated iron uptake system under iron deplete and replete conditions in \textit{S. meliloti} 2011. In this experiment, the housekeeping gene chosen was \textit{npt}.

\textbf{Fig 4.25: In vivo analysis of the iron regulation of \textit{rhbA} and \textit{rhtA} by Real-Time PCR}

\textit{(Fe+)}: Iron replete condition \textit{(Fe-)}: Iron deplete condition

The real-time RT-PCR (Fig 4.25) confirmed that the expression of the biosynthesis gene \textit{rhbA} and the gene encoding the rhizobactin 1021 outer membrane receptor, \textit{rhtA} are iron regulated.
4.6.3 The role of RirA in regulation of the iron response

Following confirmation of iron regulation of selected genes, it was decided to analyse the expression of genes involved in iron acquisition regarding potential RirA regulation.

In view of the results of chapter 3 where it was shown that the siderophore mediated iron acquisition system is not regulated by Fur, the expression of *rhbA*, a gene involved in the biosynthesis of rhizobactin and of *rhtA*, its outer membrane receptor were compared between *S. meliloti* 2011 and 2011*rirA2* using 16S rRNA as the house keeping gene.

**Fig 4.26: In vivo analysis of RirA regulation of *rhbA* and *rhtA* in *S. meliloti* 2011 by Real-Time PCR**

As shown in Fig 4.26, *rhbA* and *rhtA* are clearly down regulated by the RirA protein under iron replete conditions. The results of the real-time RT PCR shows respectively a 15 and 33-fold decrease of the expression of the genes in the wild type under iron replete conditions compared to the *rirA2* mutant.

Using the same conditions as for *rhbA* and *rhtA*, the expression of *dppA1* was analysed regarding a possible RirA regulation. The gene denoted *dppA1* is situated immediately downstream of the *rirA* gene and encodes a homologue of
an ABC transporter involved in heme transport (Carter et al., 1992). Because transcriptional regulators often regulate genes adjacent to them, the expression of dppA1 was compared between S. meliloti 2011 and 2011rirA2. Also, chapter 3 results demonstrated that Fur is not regulating smc02726, the gene encoding the heme receptor, the expression of the gene was also compared between the wild type and the rirA2 mutant.

![Graph showing expression levels of smc02726 and dppA1 in S. meliloti 2011 and 2011rirA2](image)

**Fig 4.27: In vivo analysis of RirA regulation of dppA1 and smc02726 in S. meliloti 2011 by Real-Time PCR**

The result shown in Fig 4.27 showed that under iron replete conditions, smc02726 is down regulated by RirA with a 24-fold decrease in the wild type compared to the rirA2 mutant. The fact that RirA regulates genes involved in siderophore-mediated iron uptake system and in the heme iron uptake system suggests that RirA can be the general iron regulator of S. meliloti. However, the results for the dppA1 gene were unexpected. The iron ABC transporter homologue dppA1, which is adjacent to rirA, is up regulated under iron replete conditions by the regulator. This suggested that RirA, in some cases is a negative transcriptional regulator while in the case of dppA1 is a positive regulator.
4.6.4 Regulation of RhrA as detected by real time RT-PCR

Given the known role of RhrA in regulating the siderophore mediated iron uptake system (Lynch et al., 2001), the effects of iron on the expression of \textit{rhrA} itself were determined. To do this, a real-time RT-PCR was performed comparing the level of mRNA of \textit{rhrA} in \textit{S. meliloti} under iron deplete and replete conditions. In this experiment, the housekeeping gene chosen was \textit{npt}.

\textbf{Fig 4.28: Analysis of the iron regulation of \textit{rhrA} by Real-Time PCR}

(Fe\textsuperscript{+}): Iron replete condition (Fe\textsuperscript{-}): Iron deplete condition

The real-time RT-PCR (Fig 4.28) showed that the \textit{rhrA} transcript is present under iron replete conditions. Surprisingly, the expression of the gene appears to be iron regulated with a higher concentration of transcript under iron replete conditions compared to iron deplete conditions. The expression of the gene is down regulated under iron deplete conditions with a 3-fold decrease.
4.6.5 Transcriptional regulation by RhrA under iron deplete conditions

Using real-time RT-PCR, with npt as the housekeeping gene, it was also possible to investigate and confirm the role of RhrA as a transcriptional activator comparing the level of transcripts of rhbA and rhtA in the wild type compared to the rhrA mutant under iron deplete conditions.

Fig 4.29: In vivo analysis of RhrA regulation of rhbA and rhtA in S. meliloti 2011 by Real-Time PCR

The real-time RT-PCR results confirm the regulation by RhrA of the biosynthesis gene rhbA of rhizobactin 1021 and of its outer membrane receptor gene rhtA as was suggested by RNase protection assays (Lynch et al., 2001). The only difference in the two results is that, as detected by real time RT-PCR, the transcription of the siderophore biosynthesis gene and its outer membrane receptor gene are not completely abolished under iron replete conditions.
4.7 DNA binding by RhrA

A mobility shift assay was performed with the aim of investigating the binding of RhrA to the upstream region of the rhizobactin 1021 biosynthesis genes and of the outer membrane receptor encoded by \textit{rhtA}, results that were already suggested by the real-time RT-PCR assay.

The first step was to overproduce the AraC-transcriptional regulator. Protein purification was not attempted due to the poor yield of protein obtained as a result of the poor stability and solubility of this family of transcriptional regulator. The protein extracts were therefore used to perform the mobility shift assay.

4.7.1 Cloning and expression of RhrA

The \textit{S. meliloti} \textit{rhrA} gene, which encodes a 35 KDa protein, was cloned using the same approach as for the \textit{S. meliloti} \textit{fur} homologue (Chapter 3). The gene was amplified by PCR from genomic DNA from \textit{S. meliloti} 2011. \textit{Ncol} and \textit{BamHI} sites were incorporated into the forward and reverse primers respectively. The amplified fragment extends from the start codon of \textit{rhrA} to the codon before the termination codon of this gene. This 950-bp fragment generated by PCR was cloned into the pCR2.1 vector. The \textit{Ncol-BamHI} fragment carrying the entire PCR-generated fragment was subsequently subcloned into the expression vector pQE60. This recombinant plasmid, designated pRhrA60, was used to transform \textit{E. coli} XL10 gold and Rosetta Blue.

The strategy of the \textit{rhrA} cloning into pQE60 is summarised in Fig 4.30.
Fig 4.30: Strategy of the *rhrA* cloning into pQE60
The conditions of the PCR reaction to amplify the *rhrA* gene were as follows:

**Primers:**
- **RhrA60-F:**
  CCATGG AGACAATCCGACCG
- **RhrA60-R:**
  GGATCCAGCGGCGGCTGCCAG

The PCR program used is summarised in Table 4.6.

Table 4.6: PCR Reaction Conditions for the amplification of the *S. meliloti rhrA* gene.

<table>
<thead>
<tr>
<th>PCR Conditions</th>
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<tbody>
<tr>
<td>Annealing Temperature 55°C</td>
</tr>
<tr>
<td>Annealing Time 1 min</td>
</tr>
<tr>
<td>Extension Time 72°C for 1 min</td>
</tr>
</tbody>
</table>
Problems were encountered with the over expression of \textit{rhrA} probably because of the properties of this protein, which is a member of a family of proteins renowned for being insoluble and unstable. To solve the difficulty of insolubility, a lower growth temperature of 30°C was used. Indeed, when produced under optimum growth conditions, the overexpression of RhrA gave rise to the formation of inclusion bodies, which are formed through the accumulation of folded intermediates. The use of 30°C temperature permitted the protein to fold properly and thus to reduce the formation of inclusion bodies. Also, the strain \textit{E. coli} Rosetta Blue was used as it is designed to enhance the expression of proteins that contain codons rarely used in \textit{E. coli}. It supplies tRNAs for 6 rare codons, AUA, AGG, AGA, CUA, CCC, GGA, on a compatible chloramphenicol-resistant plasmid called \textit{pRARE}. An analysis of \textit{rhrA} regarding codon usage indicated that it possesses 8.3% of rare codons, which is quite above the average (Novy et al., 1999). The yield of RhrA obtained with XL10 gold even under denaturing conditions was low and thus Rosetta Blue was used as an alternative. Also, time courses were undertaken to optimise the temperature of growth and the length of growth time after induction with IPTG. Gradients of different lengths of sonication (important due to the poor stability of the protein) and different concentrations of IPTG were used to determine the optimal conditions for high yields of protein.

It was determined that the optimised protocol for the overexpression of RhrA was as follows. \textit{E. coli} Rosetta Blue carrying \textit{pRhrA60} was inoculated into LB containing 100 µg/ml of ampicillin (to maintain the \textit{pRhrA60} plasmid) and 30 µg/ml of chloramphenicol (to maintain the \textit{pRARE} plasmid) and were grown at 30°C until the \text{OD}_{600} reaches 0.4 to 0.6. IPTG was added to a final concentration of 0.05 mM, and the cultures were grown for an additional 6 hours. A 1.5 ml aliquot of the cells was then pelleted and resuspended in 250 µl of lysis buffer as described in chapter 2. The cells were kept on ice and were sonicated for 20 seconds. Finally, the cellular debris was pelleted at 10,000 rpm for 10 minutes and the remaining supernatant was stored at -20°C.
Fig 4.31 shows the results of a time course experiment of the protein extract prepared under denaturing conditions and expressed under optimal conditions. It was concluded that extract should be harvested six hours after induction.

Fig 4.31: 15% SDS polyacrylamide gel electrophoresis of extract of cells overexpressing RhrA prepared under denaturing conditions

Lane 1: Ladder
Lane 2: Non-induced at time 0
Lane 3: Induced at time 0
Lane 4: Non-induced after 2 hours
Lane 5: Induced after 2 hours
Lane 6: Non-induced after 4 hours
Lane 7: Induced after 4 hours
Lane 8: Non-induced after 6 hours
Lane 9: Induced after 6 hours
Lane 8: Non-induced after 8 hours
Lane 9: Induced after 8 hours
Fig 4.32 shows RhrA prepared under native conditions from cells grown under optimal conditions. The protein looks slightly bigger on the gel than its 35 KDa due to the 6xHis-tagged fused to its C-terminal. A densitometry analysis has shown that the native RhrA represents 4.8% of the total protein content. Ultimately, the His tag was not exploited for protein purification.

Fig 4.32: 15% SDS polyacrylamide gel electrophoresis of extracts containing RhrA protein that had been prepared under native conditions

Lane 1: ladder
Lane 2: pRhrA60 non-induced
Lane 3: pRhrA60 induced
4.7.2 Mobility Shift Assay using protein extracts containing overexpressed RhrA

The RhrA mobility shift assay was undertaken using the region upstream of rhtX and the rhrA-rhtA intergenic region with extracts from E. coli Rosetta Blue containing pRhrA60.

The synthesis of the DNA probes and their labelling was performed in the same way as for the mobility shift assay carried out for the S. meliloti recombinant Fur. Two of the probes used for the Fur EMSA were used in this experiment (See Chapter 3):

**Probe 1: Intergenic sequence between rhtX and the open reading frame of orf2**

```
CGGGATCCCATGCGGCTCTCTCGAAATGCGTTCGCTACTGTCT
TAATGACTTCGCTCACATCAGGCGTTCCACGACGTCATTT
AAAGATGAGCGCAACTCTATGTATTATCGTCAGACATGTGCTCC
GGCAGTCGAGTCTTTCGGGATCCCG
```

Highlighted in orange are the putative RhrA repeat binding sites and underlined, the 6-bp repeat present upstream rhtX and in the intergenic region rhrA-rhtA and purple the BamHI sites used in labelling the probe.

**Probe 2: Intergenic sequence between rhrA and rhtA**

```
CGGGATTCGTGCGGCGCGAGCGCTTCTCTGTGACGTTCGCATGCG
TCCAATGAGTTTCGCATTACAAGCGCGAGACACCCCTTACCC
CATAAAAATGACTTAAATATTTGTATTTGGCAATTTGCGCGCC
CACCAGCAGCGCAATGTATCTGTGTCGCAGGGGCGTTATG
GGCAGGATCCCG
```

Highlighted in orange are the putative RhrA repeat binding sites and underlined, the 6-bp repeat present upstream rhtX and in the intergenic region rhrA-rhtA and purple the BamHI sites used in labelling the probe.
The conditions of the mobility shift assay were as described in chapter 2.

The negative controls, which are the DNA probes on their own in the binding buffer or the probes in the binding buffer but also with an *E. coli* extract with pQE60 induced, showed no band shift (Fig. 4.33: lane 1,2,3 and 5).

However, in Fig 4.33, in the lane 4 and 6, which are the probes with extracts of *E. coli* with pRhrA60 induced, a band shift can be observed showing physical evidence of RhrA binding to the region upstream *rhtX* (Lane4) and in the intergenic region between *rhrA* and *rhtA* (Lane 6).
The mobility shift assay provided physical evidence of the binding of RhrA to the promoter region of *rhtXrhbABCDEF* and in the intergenic region between *rhrA* and *rhtA*
4.8 Effect of the *rirA2* mutation on symbiotic performance

*S. meliloti* 2011 induces nodule formation and enters into a nitrogen fixing symbiosis with *Medicago sativa* (alfalfa). The effect of the *rirA* mutation in the mutant strain 2011*rirA2* on plants was examined. The effect of *S. meliloti* 2011 on plants was examined as a positive control. Uninoculated plants were examined as a negative control.

With the help of Dr. O Cuiv, following a thirty-day incubation, the plants were analysed to determine if the mutants had nodulated. All the plants examined showed nodule formation. The nodules had a reddish hue indicating the presence of leghaemoglobin. The nodules were similar to those produced by *S. meliloti* 2011. The uninoculated plants did not show any nodule formation. No difference was observed between plants indicating that the *rirA* mutation was not having a noticeable effect on symbiosis.
4.9 Discussion

Chapter 4 results suggest that in *S. meliloti*, a new type of transcriptional regulator denoted RirA regulates the acquisition of iron. This is the second member of this Rhizobial Iron Regulator (RirA) family discovered after the one found in *R. leguminosarum* by Todd *et al.* (2002)

Previously, it was concluded in chapter three that Fur, which is the main general iron regulator in gram-negative bacteria, did not fulfil this function in *S. meliloti*. In addition, no homologue of DtxR, the other general bacterial iron regulator found in bacteria, was found in *S. meliloti*. Thus, the work was directed to the homologue of RirA from *R. leguminosarum*, which was identified in *S. meliloti* by blast analysis with a high homology of 88%

*S. meliloti* RirA shows a lot of homology to the protein family denoted Rrf2. To date, there has been little study of this family of transcriptional regulators. They have a helix turn helix motif but nothing is known about the DNA sequence they bind to and whether or not they need a cofactor. An interesting characteristic of this family is three cysteines present on the C-terminal of its members. This site could be where the ferrous iron binds to the protein and possibly acts as a cofactor.

In order to find out about its function, the gene was mutated to study its putative role in iron regulation. The siderophore plate bioassay showed that in the *rirA* mutant, the siderophore rhizobactin 1021 is constitutively produced. The result was confirmed by the use of promoter probes. They were constructed in pOT1, cloning the promoter region of *rhtXrhhABCDEF* upstream of a GFP reporter gene. The different constructs were mobilised into the *S. meliloti* 2011 wild type but also in 2011*rirA2*. When comparing the GFP activity emitted by pWT, carrying the intact promoter region, mobilised into 2011 and into 2011*rirA2* under iron replete conditions, some GFP activity was observed solely in the *rirA* mutant suggesting that in the presence of iron, RirA down regulates at the transcriptional level the expression of the siderophore. This may occur by
binding to the promoter region of the rhizobactin biosynthesis operon. Also, a
35-bp DNA sequence was identified to be necessary for the action of RirA. Its
deletion results in the constitutive expression of GFP. To find out more about the
genes regulated by RirA, analysis of the transcription of iron responsive genes
were performed with the help of real-time RT-PCR. rhbA and rhtA were found to
be iron regulated through RirA. Thus, RirA is the repressor of the siderophore
mediated iron uptake system. These results are similar to the ones obtained in R
leguminosarum, in which biosynthesis of the siderophore vicibactin and its outer
membrane receptor are down regulated by RirA under iron replete conditions.

In view of the results of chapter 3, the regulation by RirA of the heme receptor
encoded by smc02726 was assessed. Because the gene adjacent to rirA, dppA1
encodes an iron transporter protein homologue, its regulation was also examined.
The heme transporter was found to be down regulated by RirA under iron replete
conditions, a result that is similar to the one observed in R leguminosarum
where the genes involved in heme uptake (hmu and tonB) are regulated by RirA
Furthermore, the iron responsive expression of smc02726 was observed in S
meliloti Rm818 (unpublished data), a strain that is cured of the pSymA
megaplasmid and therefore lacks rhrA, the gene encoding the AraC-like activator
of the rhizobactin 1021 biosynthesis and transport genes. This result is significant
in that it decouples the iron responsive activity of rirA from the effect of RhrA.
On the other hand, surprisingly, dppA1 was found to be up regulated. This result
shows that as well as being a repressor, RirA can also act as a positive regulator.

For RirA up regulation, RirA could act either directly or indirectly, as is the case
for Fur. For example, the ferric uptake regulator Fur is mainly known to act as a
negative transcriptional regulator, however, recently, it was shown to also act as
a positive transcriptional regulator (Delany et al., 2004). In some cases, Fur also
indirectly up regulates the expression of genes via a small RNA ryhB, which
itself negatively regulates genes at the posttranscriptional level.

The real-time RT-PCR experiments have shown that RirA is iron regulated and
abundantly present under iron replete conditions. One hypothesis is that the
regulator might autoregulate itself. Under iron replete conditions, the ferrous iron
present in the cell might bind to the molecule acting as a cofactor. This alteration of conformation might result in the protein binding to its promoter region and upregulating its expression. On the other hand, under iron deplete conditions, because of the absence of ferrous iron, a different conformation of the regulator appears and thus, the expression of \textit{rirA} is considerably decreased. As previously mentioned, this change of conformation could happen by the iron binding to the 3 cysteines present of the C-terminal. This is, for example, the case for the regulatory functioning of FNR, the transcriptional regulator of anaerobic respiration of \textit{E. coli}. Indeed, the interconversion of both forms of the protein appears to be regulated by the availability of O\textsubscript{2} but also by the binding of ferrous iron to the cysteine residues (Trageser \textit{et al}, 1989).

It is also likely that the regulator acts in the same way as the Fur and DtxR proteins down regulating genes directly by binding to their promoter regions. Indeed, the fact that the regulation of \textit{RirA} is not restricted to the siderophore system suggests that \textit{RirA} is a general iron regulator, like those proteins. \textit{RirA} also appears to be the general iron response regulator in \textit{R. leguminosarum} (Todd \textit{et al}, 2002). In contrast, in \textit{B. japonicum}, it has been found that an additional protein, Irr, functions along with Fur in the iron response (Hamza \textit{et al}, 2000). There is no obvious reason why some rhizobia have recruited \textit{RirA} as an alternative to Fur as the general iron response regulator.

A parallel investigation was undertaken of \textit{RhrA}, an AraC-transcriptional activator encoded downstream from the siderophore biosynthesis genes and upstream from the outer membrane receptor genes. Similar sets of repeats were identified upstream from \textit{rhtXrhbABCDEF} and in the intergenic region of \textit{rhrA-rhtA} (Fig 4.34), two promoters known to be activated by \textit{RhrA} (Lynch \textit{et al}, 2001).
**Region upstream rhtX:**

```
GCGTCGCTACTGTCTTAATGAGGTCGTC
```

**Intergenic region rhrA-rhtA:**

```
ACGTCGCTACTGTCTCAATGAGGTCGCA
```

**Fig 4.34:** Region of the putative RhrA binding repeats upstream from rhtX and in the intergenic region of rhrA-rhtA.

The binding sites are framed and the 6-bp additional repeats between the two regions are underlined.

The results of the levels of GFP activity suggested that in *S. meliloti*, under iron deplete conditions, the expression of the operon *rhtXrhhABCDEF* and thus the production of the siderophore rhizobactin 1021 is dependent on the presence of the proximal repeat in the promoter region of the biosynthesis cluster. Also, no GFP activity was detected for *S. meliloti* 2011 [pENT4] even if the proximal repeat was present but moved by the insertion of a *BglII* site. Interestingly, both putative RhrA repeats can be found in the intergenic region between *rhrA* and *rhtA*. Additionally, the 6-bp upstream the proximal repeat upstream from *rhtX* and the 6-bp of the proximal repeat upstream from *rhtA* are identical (Fig 4.34). In pEN4, a *BglII* site was inserted between those 6 bases and the proximal repeat upstream from *rhtX*. It is thus possible that this 6-bp are involved in the binding of RhrA and the disruption of the continuity between the 6-bp sequence underlined in Fig 4.34 and the proximal repeat in pEN4 abolishes the binding site of RhrA.

Analysis of the mRNA of *rhhA* and *rhtA* showed that the transcriptional regulator is strongly involved in their regulation, up regulating their expression under iron deplete conditions. These results confirmed the RNAse protection assays carried out on these genes (*Lynch et al.*, 2001). The only difference between those results is that *Lynch et al.* (2001) results concluded that under iron replete conditions no transcripts of those genes could be detected which differs from the
results from the real-time RT-PCR assays. One explanation is that under iron replete conditions, the cell still needs to balance its iron concentration and while it is abundantly available in the environment, it might still produce a minor amount of siderophore to take up the amount of iron the cell uses in redox reactions and in the production of proteins and enzymes.

Some results observed with RhrA are similar to the ones observed with PchR, YbtA and AlcR. In the four cases, the siderophore biosynthesis genes and outer membrane receptors are regulated by AraC-like regulators under iron deplete conditions. Also, as is the case for YbtA, the presence of the proximal repeat seems to be crucial for the binding of the transcriptional regulator. However, some differences can be observed. PchR, YbtA and AlcR activation were reported to be siderophore dependent or partially dependent in all cases. However, Dr O Cuiv, a member of this group has shown that under iron deplete conditions, in an *S. meliloti* siderophore biosynthesis mutant, the outer membrane receptor RhtA is still expressed. This clearly show that the activation of RhrA is not dependent on the presence of rhizobactin 1021. Also, at least in two cases, for YbtA and PchR, the regulator is also able to negatively autoregulate itself. However, from the results of this chapter, contrary to these two AraC-like regulators, RhrA seems to be more abundant under iron replete conditions.

The overall aim of this thesis was to determine how *S. meliloti* responds to changes in iron availability, and in particular, how *S. meliloti* regulates the siderophore mediated iron uptake system. Our analysis of iron-dependent gene expression in *S. meliloti* has revealed that the rhizobactin 1021 biosynthesis genes, as well its outer membrane receptor, whose expression was in each case recognised to be iron regulated are regulated, by a regulatory mechanism involving both RirA and RhrA. If RirA is solely present under iron replete conditions, RhrA is present under both iron replete and deplete conditions. One possible mechanism is that by binding to the promoter region of iron-regulated genes, RirA prevents the binding of RhrA. Indeed, if a 35-bp DNA sequence was identified as where RirA binds, however, there is no evidence that this is the complete binding sequence or that RirA acts as a monomer and not a multimer. It is thus possible that RirA could prevent the binding of RhrA binding to the
sequence at its proximal repeat, which appears to be the most important. Under iron replete conditions, ferrous iron could bind to RirA putatively on the 3 cysteines present of its C-terminal and so positively auto regulating the expression of its gene while the absence of iron could lead to a change in the conformation of RirA that would then be down regulated. In this case, with the cell being deficient in RirA, RhrA could bind to the promoter regions of rhtXrhhABCDEF and rhtA provoking the positively regulation of those genes.
Chapter 5:

Luteolin regulation of the siderophore biosynthesis gene *rhhG* in *Sinorhizobium meliloti*
5.1 Introduction

*S. meliloti* is an agriculturally important soil bacterium that is capable of forming a nitrogen-fixing symbiosis with leguminous plant host alfalfa (*Medicago truncatula*). The exchange of molecular signals between the host and the bacterium controls the nodulation process by which *S. meliloti* invade the plant roots. Flavonoids, which are released by plants and which accumulate in the rhizosphere are the first of those signals. More than 4000 different flavonoids have been identified in plants, and a particular subset of them is involved in mediating host specificity in the legumes (Perret *et al.*, 2000). The flavonoid specific to alfalfa that function as a signal to *S. meliloti* is luteolin (Fig 5.1).

![Chemical structure of luteolin](image)

**Fig 5.1: Chemical structure of luteolin, the inducer released from *M. truncatula***

The proposed mechanism involves luteolin diffusing into the bacteria where it interacts with NodD proteins, which are members of the LysR family of transcriptional regulators. The flavonoid then triggers a signal transduction cascade that controls the infection process (Broughton *et al.*, 2000; Perret *et al.*, 2000). Even in the absence of flavonoids, tetrameric NodD binds to a conserved 49 bp motif (Nod-box) that is found in the promoters of nodulation (*nod, nol* and * noe*) genes (Feng *et al.*, 2003). Nevertheless, compatible flavonoids are required
for the activation of *nod*-loci (Fisher and Long, 1993) Most nodulation genes that are expressed in a flavonoid- and NodD-dependent manner are involved in the synthesis of strain-specific lipochito-oligosaccharides called Nod-factors that are essential for the initial infection of root-hairs by the bacteria. Although flavonoids and Nod-factors represent the first set of molecular signals exchanged, other signals are required for successful invasion of the host and ultimately differentiation of infecting rhizobia into functional nitrogen-fixing bacteroids (Broughton *et al.*, 2000, Perret *et al.*, 2000).

Recent literature has established that surprisingly, flavonoids could affect the expression of several genes, which are not among the *nod* genes and do not possess a ‘Nod box’ in their promoter regions (Perret *et al.*, 1999, Chen *et al.* 2000). A short time ago, Ampe *et al.* (2003) reported with the help of macroarrays that five *S. meliloti* genes involved in iron metabolism are significantly induced by luteolin. Interestingly, one of those genes are related to iron metabolism, *rhbG* which is a gene thought to be involved in the biosynthesis of the *S. meliloti* siderophore (Lynch *et al.*, 2001). *rhbG* is located distal to the *rhtA* gene in the rhizobactin 1021 regulon (Fig 3.1 in chapter 3).

It was decided to investigate further the regulation of *rhbG*, which based on bioinformatics analysis may be coding for the lipid tail of rhizobactin 1021. Therefore, the activity of an *rhbG lac* fusion was investigated under iron deplete and replete conditions and with and without luteolin to determine how the expression of this gene is controlled.
5.2 \textit{in vivo} analysis of the luteolin regulation of \textit{rhbG} under iron deplete and replete conditions.

An \textit{rhbG-Tn5lacZ} mutant strain was previously generated in our laboratory by transposon insertion (Lynch, PhD thesis, 1999) and called \textit{2011rhbG25}. This strain carries the Tn5 \textit{lacZ} transposon inserted in the chromosomal copy of the \textit{rhbG} gene in the correct orientation. The mutant was constructed using \textit{S. meliloti} G212, deletion mutant of \textit{S. meliloti} 2011 (Glazebrook et al., 1989). β-galactosidase assays were performed as described in chapter 2, to determine the expression of the gene under different conditions of growth. \textit{S. meliloti} G212 was used as a negative control. The bacteria were grown in TY media under iron deplete and replete conditions and in both cases in the absence and in the presence of luteolin (The concentrations of 2,2'-dipyridyl was 300 μM and of luteolin 10μM). When added with luteolin, which is prepared in methanol, 2,2'-dipyridyl was resuspended in methanol instead of ethanol, as the addition of the two solvents to the media resulted in the appearance of a precipitate.
A β-galactosidase assay was carried out according to the Miller protocol (1972) with some modifications based on Mulligan et al. (1985) whereby when no yellow colour appear, the reaction was stopped after 20 minutes.

Table 5.1: β-galactosidase activity results of S. meliloti G212 and S. meliloti G212rhhbG25 under iron deplete and replete conditions and in the absence and presence of the inducer, luteolin.

<table>
<thead>
<tr>
<th></th>
<th>β-galactosidase activity (Miller Unit)</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron replete conditions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. meliloti G212</td>
<td>3.3</td>
<td>+/- 3.3</td>
</tr>
<tr>
<td>S. meliloti G212rhhbG25</td>
<td>11.61</td>
<td>+/- 1.41</td>
</tr>
<tr>
<td>Iron replete conditions with the addition of luteolin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. meliloti G212</td>
<td>1.81</td>
<td>+/- 0.10</td>
</tr>
<tr>
<td>S. meliloti G212rhhbG25</td>
<td>253.50</td>
<td>+/- 2.73</td>
</tr>
<tr>
<td>Iron deplete conditions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. meliloti G212</td>
<td>3.50</td>
<td>+/- 0.69</td>
</tr>
<tr>
<td>S. meliloti G212rhhbG25</td>
<td>381.69</td>
<td>+/- 93.90</td>
</tr>
<tr>
<td>Iron deplete conditions with the addition of luteolin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. meliloti G212</td>
<td>2.92</td>
<td>+/- 0.10</td>
</tr>
<tr>
<td>S. meliloti G212rhhbG25</td>
<td>763.12</td>
<td>+/- 61.64</td>
</tr>
</tbody>
</table>

The basal level of endogenous β-galactosidase activity in the S. meliloti strain G212 is, as expected, low and is not affected by the plant exudates or by the different iron conditions.

A low level of expression was detected in S. meliloti 2011rhhbG25 under iron replete conditions but a 33-fold expression increase was seen under iron deplete conditions (Table 5.1). This implies that rhhbG is iron regulated.

Also, under iron replete conditions, the behaviour of the β-galactosidase activity was examined in the presence of luteolin. The level of β-galactosidase activity in the mutant S. meliloti strain 2011rhhbG25 is also relatively low but is considerably increased in the presence of luteolin. When comparing the expression levels with and without luteolin, a 22-fold expression increase can be detected in the presence of luteolin (Fig 5.2).
Fig 5.2: β-galactosidase activity (Miller U) under iron replete conditions in the presence and absence of luteolin of G212 and G212rhhG25.

The same comparison done under iron deplete conditions shows that the level of expression is also increased in the presence of luteolin but only 2-fold (Fig 5.3). These results suggest that the expression of rhhG is under positive regulation by luteolin and that this regulation is achieved through a complex and unknown mechanism. Luteolin usually regulates genes indirectly through NodD but no ‘Nod box’ was detected in the promoter region of rhhG.
Fig 5.3: β-galactosidase activity (Miller U) under iron deplete condition in the presence and absence of luteolin of G212 and G212rhbG25.
5.3 Influence of the *rhbG25* mutation on symbiotic performance

The effect of the *rhbG* mutation in the mutant strain G212*rhbG25* on *Medicago sativa* (alfalfa) plants was examined. Inoculation of plants with *S. meliloti* 2011 was examined as a positive control. Uninoculated plants were examined as a negative control. Plant tests were carried out with the help of Dr. Ó Cuív.

Following a thirty-day incubation, the plants were analysed to determine if the mutants had nodulated. All the plants examined showed nodule formation. The nodules had a reddish hue indicating the presence of leghaemoglobin. The nodules were similar to those produced by *S. meliloti* 2011. The uninoculated plants did not show any nodule formation. No difference was observed between plants indicating that the *rhbG* mutation was not having a noticeable effect on symbiosis.
5.4 Discussion

The results of this chapter clearly showed the iron and luteolin regulation of \( rhbG \)

Based on bioinformatics analysis, the gene is thought to encode the lipid tail of the rhizobactin 1021 siderophore and thus the iron response of its expression was expected, as biosynthesis of the siderophore is upregulated under iron deprivation. In addition, the plant test showed that the expression of \( rhbG \) is not crucial for plant nodulation. This observation concurs with the results observed with siderophore biosynthesis mutants for which no difference was observed in nitrogen-fixing ability in acetylene reduction was observed compared to the wild type (Lynch et al., 2001). Competition studies between \( S. meliloti \) 2011 and \( 2011rhbG25 \) would allow an assessment of the importance of the lipid tail encoded by \( rhbG \).

It is also very interesting to observe a significant increase in the expression of \( rhbG \) under iron replete conditions and to a more moderate extent under iron deplete conditions in the presence of the plant flavonoid luteolin which confirms the macroarray results from Ampe et al. (2003). The difference in fold increase in \( rhbG \) could be that the limit of rhizobactin 1021 expression in the presence of the flavonoid under iron deprivation was reached leading to a smaller fold increase compared to the expression under iron replete conditions.

To date, the best understood signalling function of flavonoids involves the transcriptional regulation of the \( nod \) genes in \( S. meliloti \), which possesses three \( nodD \) genes, the proteins NodD1 and NodD2 are known to be activated by luteolin and to bind the ‘Nod box’ but analysis of the promoter region of \( rhbG \) did not show any ‘Nod box’.

However, some isoflavonoids are known to play molecular roles beyond the enhancement of \( nod \)-gene transcription. For example, daidzdem regulates the expression of two genetic loci that are apparently unrelated to \( nod \) genes in
Rhizobium fredii (Sadowsky et al., 1988) Also, it has been established that flavonoids could affect the expression of genes, which are not the nod genes and without the presence of a ‘Nod box’ in their promoter regions (Perret et al., 1999, Chen et al. 2000) and it seems to also be the case that luteolin affects genes that are not directly involved in nodulation.

The reason for the involvement of luteolin in iron acquisition is unclear. However, it is known that interactions can occur between bacteria and plant roots that can be beneficial to the plant. For instance, plants can profit from bacterially induced growth promotion and protection against pathogens.

Studies have shown that luteolin release from alfalfa induces a positive chemotaxis in S. meliloti (Caetano-Anolles et al., 1988, Dharmatilake et al., 1992). Also, the flavonoids, luteolin and quercetin, have a very definite promotive effect on growth of S. meliloti in a minimal medium (Hartwig et al., 1991). One way to promote this growth could be by promoting an increase in the expression of the siderophore rhizobactin 1021 under iron replete and deplete conditions. The increase in the production of rhizobactin 1021 would make S. meliloti more competitive and thus present in higher concentration leading to a more efficient symbiotic relationship between S. meliloti and alfalfa.

Also, if the bacterium grows better, it could compete more efficiently with other organisms such as pathogens. Previous studies show that siderophores can be implicated in the induction of resistance such as in Arabidopsis (Van Loon et al., 1998), in tobacco (Maurhofer et al., 1994) and in radish (Leeman et al., 1996). The rhizobactin 1021 siderophore present in high concentration could chelate the iron available and thereby deprive pathogens of essential iron. Thus, the extensive colonisation of the plant by S. meliloti could prevent pathogens from establishing themselves on or in the alfalfa.
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