Genetic regulation of Iron Responsive Genes in *Sinorhizobium meliloti*

Thesis
Presented for the Degree of
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By

Caroline Viguier B.Sc., M.Sc.

Under the supervision of
Michael O'Connell, B.Sc., Ph.D.

School Of Biotechnology, Dublin City University,
Dublin 9, Ireland
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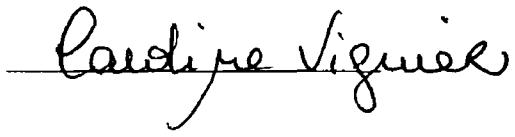
Declaration

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

Signed 

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Sans oublier mes fidèles amis Agnès, Alexis, Erwann, Didier, Anne et David

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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 *rhbG*, 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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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 $O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$ (1)

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

Haber-Weiss reaction (1) + (2) $O_2 + H_2O_2 \xrightarrow{\text{Fe catalysis}} HO + OH^- + O_2$ (3)

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

1 2 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 1 1 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 (70-100kDa) 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 (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.

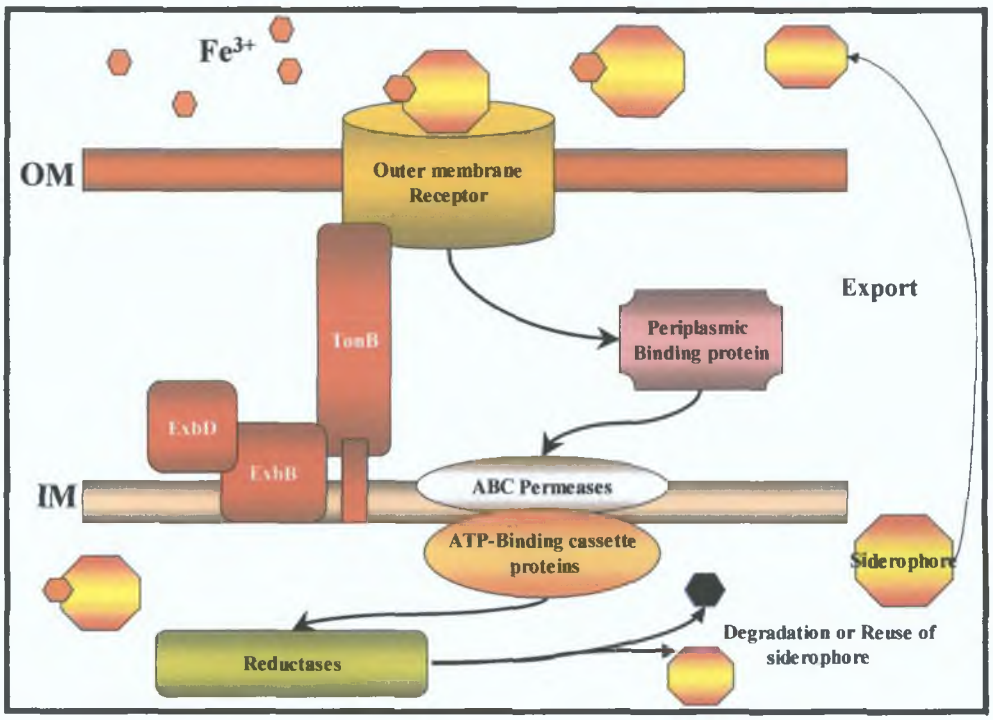


Fig 1.1: Schematic of the siderophore iron uptake system in gram-negative bacteria

In 2004, a new family of transport proteins was identified in a variety of bacteria including *Sinorhizobium meliloti* and *Pseudomonas aeruginosa* (Ó Cuív *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 Cuív *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).

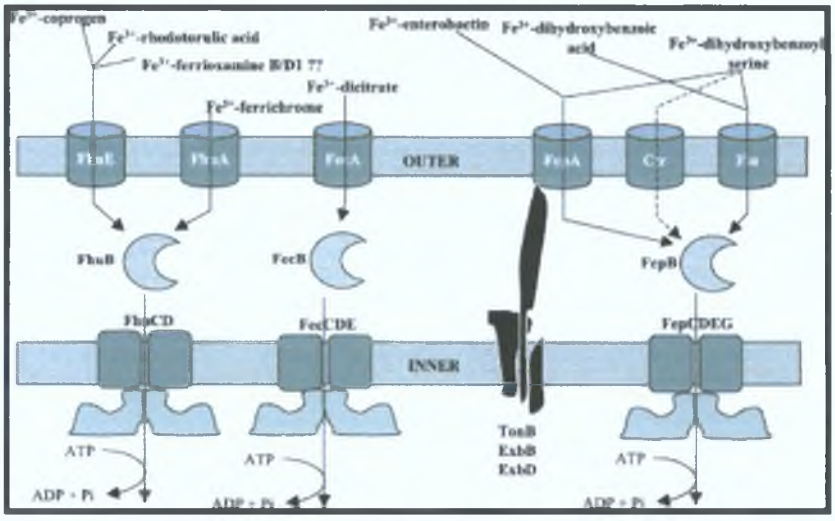


Fig 1.2: Schematic representation of siderophore-mediated iron uptake systems in *E. coli* K-12.

Note that the TonB-ExbB-ExbD complex energises and interacts with all the OM receptors shown (not just FepA). (Andrews *et al.*, 2003)

1 2 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

1 2 2 1 Iron uptake from glycoproteins

The iron binding proteins transferrin, contained in human serum, and lactoferrin,, contained in mucosoal 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

1 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

1 2 3 Fe^{2+}- transport system

Under anaerobic conditions, ferrous iron can be available To acquire it, a transport system is generated by the three genes *feoABC* in *E coli* (Kammler *et al*, 1993) *feoA* and *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 *feoA* and *feoB* showed a strongly reduced ferrous iron uptake phenotype *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, *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)

1 2 4 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)

1 2 4 1 Strategy I

Dicotyledons and non-grass monocotyledons reduce ferric iron before uptake (Fig 1 3 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.

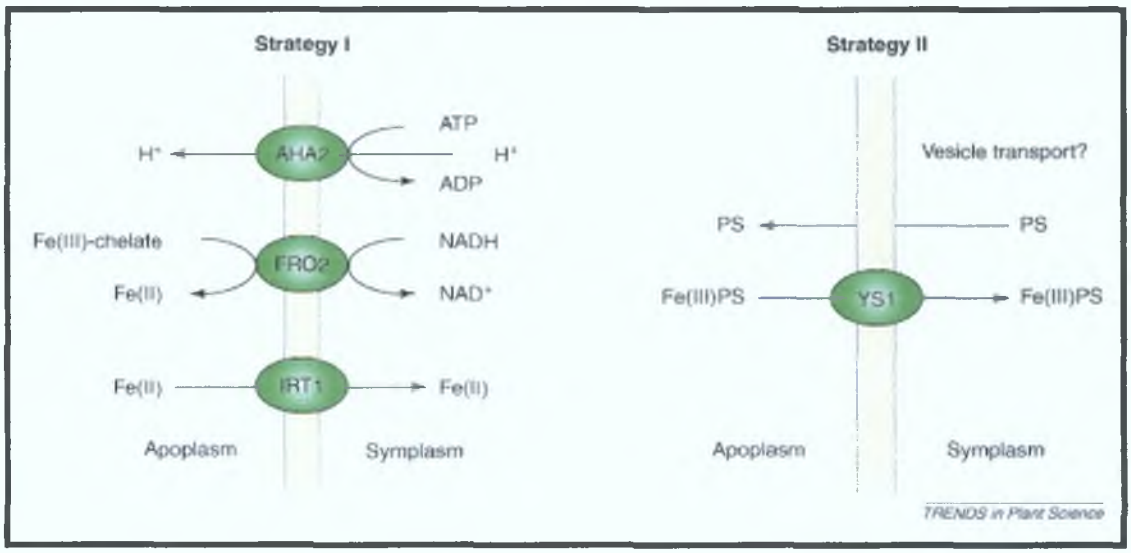


Fig 1.3. Mechanisms of iron uptake by plants.
In strategy I plants (e.g. *Arabidopsis*, pea and tomato), ferric iron chelates are reduced before the ferrous iron is transported across the plasma membrane. Strategy II plants (e.g. barley, maize and rice) release siderophores capable of solubilising external 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

1 3 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-Tehrani *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 *bfd* 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_2O_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_2O_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_2O_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_2O_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 tighly 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 approximatively 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 approximatively 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)

1 4 1 2 Fur regulon

To this point, as many as ninety genes have been found to be regulated by Fur (Fig 1 4) 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.

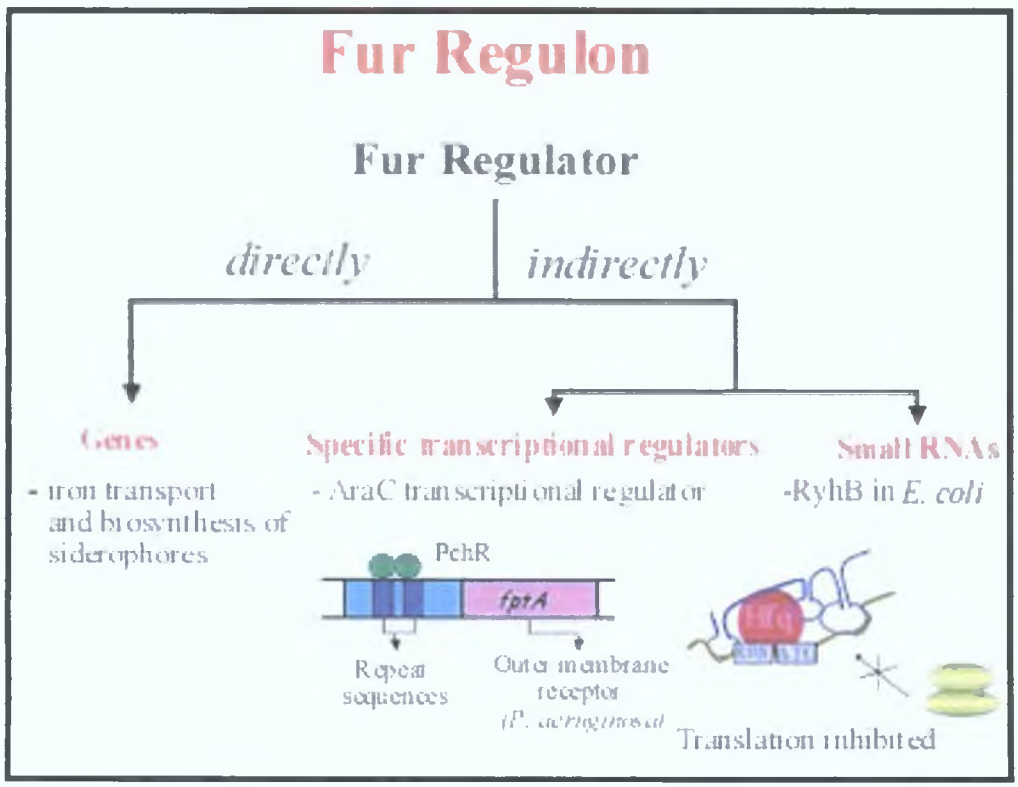


Fig 1.4: Different levels of regulation by the ferric uptake regulator Fur

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

1 4 1 3 Fur regulation

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, Stojiljkovic *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

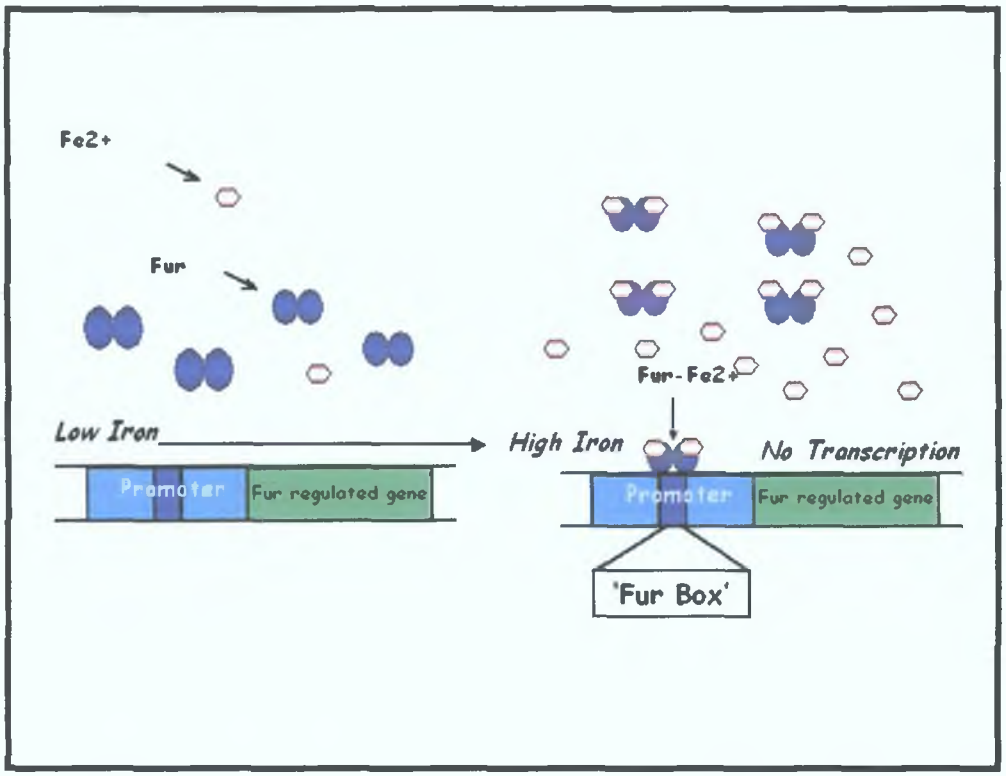


Fig 1.5: Model of regulation by the transcriptional regulator Fur

1.4.1.4 Fur binding

The purification of Fur facilitated the investigation of its activity as a DNA binding repressor *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 promotor 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

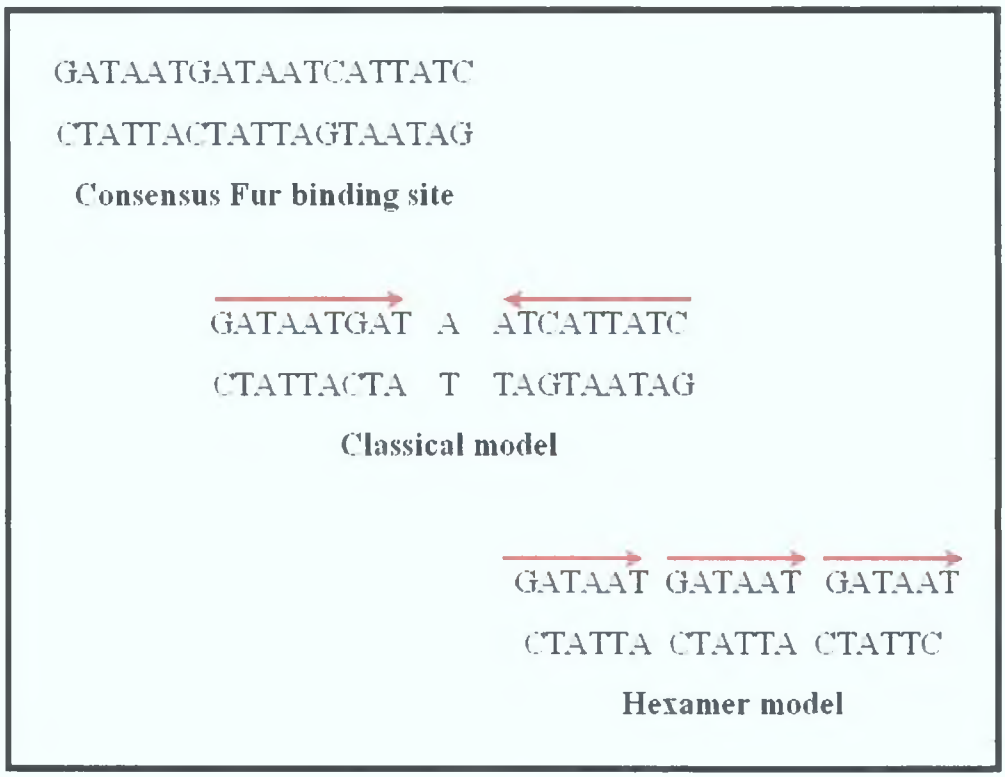


Fig 1.6: Fur binding site.
The 19-bp consensus Fur binding site for *E. coli* and various models of recognition are shown. The top sequence shows the consensus Fur binding site. The classical model shows each monomer binding a 9-bp inverted repeat (shown as arrows) of the consensus, with an A:T base 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 recognises 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.

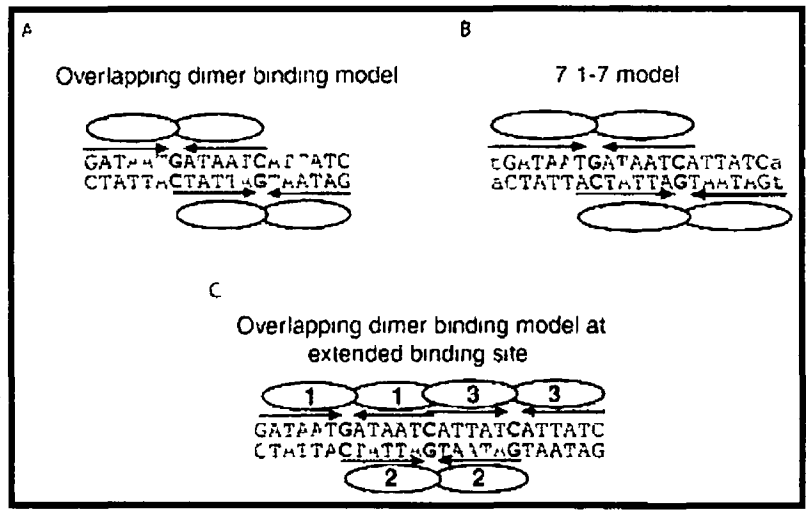


Fig 1 7 Models for Fur-DNA interactions
A represents the overlapping-dimer binding model In this model, each monomer (shown as an oval) binds an inverted hexamer, shown as an arrow, with two dimers required for binding the 19-bp consensus C G base pair spacers are shown in bold B shows the 7-1-7 model, 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)

1 4 1 5 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 $[2Fe\text{-}2S]^{2+}$ 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

1 4 1 6 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 cellular role in addition to its role in iron homeostasis Indeed, the *fur* gene mutation appears to be lethal in *Neisseria* and *V anguillarum*

1 4 1 7 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

1 4 1 8 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 diphteria 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 diphteria 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*

1 4 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*

1 4 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 Sheppart *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).

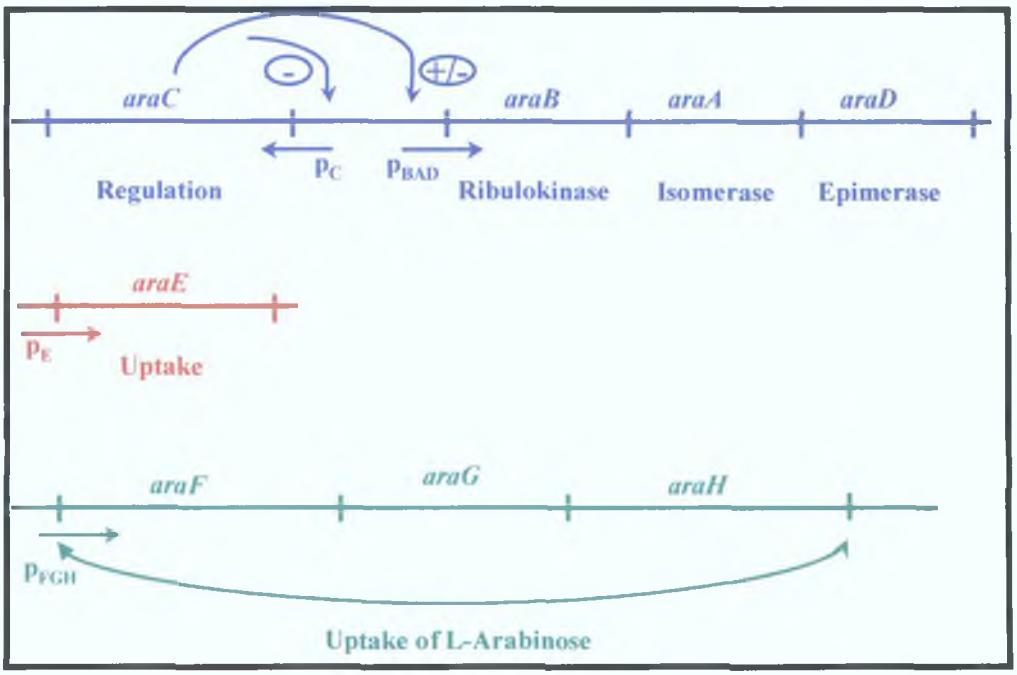


Fig 1.8: Organisation of the genes of the L-arabinose operon

1 4 2 3 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 I_1 and O_2 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 I_2

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 I_1 and I_2 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 O_2 and I_1 and part is independent of looping

1 4 2 4 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 chrystallography 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).

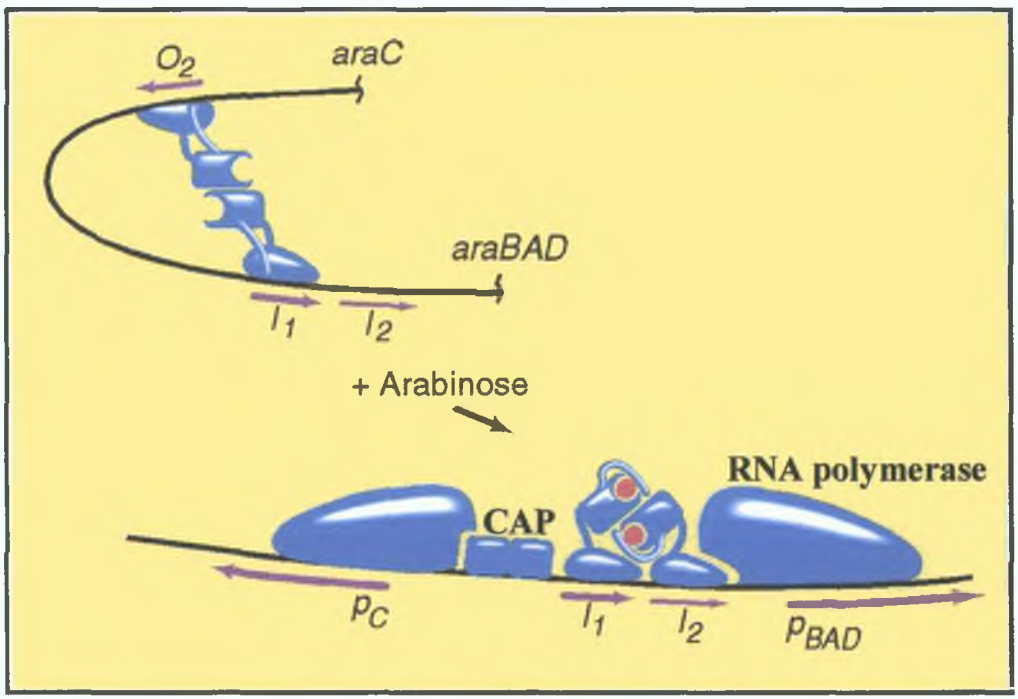


Fig 1.9: Light switch mechanism of the AraC protein in *E. coli*
Binding of AraC in *trans* to the O_2 and I_1 half-sites to form a DNA loop in the absence of arabinose and its binding *cis* to the I_1 and I_2 half sites in the presence of arabinose that leads to unlooping and induction of p_{BAD} and transient derepression of p_C and the light-switch mechanism (Schleif *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 *et al.*, 1993).

1 4 2 5 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 *alcABCDER* operon but also at the secondary promoter-operator in the *alcR* upstream region However, there is no evidence so far for negative auto regulation of AlcR

***YbtA in* Y pestis**

The second example of an AraC transcriptional regulator is YbtA in *Yersinia pestis*, the causative agent of plague Under iron deplete conditions, *Y pestis* produce a siderophore called yersiniabactin or yersiniaphore (Fetherston *et al*, 1995, Wake *et al*, 1975) This siderophore is also produced by *Y enterolitica* 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 *irp2*, which encodes a 190 kDa iron regulated high molecular weight protein called HMW2 found in yersinia species is involved (Carniel *et al*, 1989, Fetherston *et al*, 1995, Guilvout *et al*, 1993) This gene is part of what is more than likely the yersiniabactin biosynthesis operon (Carniel *et al* 1992, Fetherston *et al*, 1995) *psn* encodes the outer membrane receptor for both pesticin and yersiniabactin

YbtA controls the expression of the siderophore yersiniabactin biosynthesis protein encoded by *irp2* and the expression of its outer membrane protein encoded by *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 *irp2* decreases the expression of *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 yersiniabactin 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 *irp2* 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* (Serino *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)

1 4 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*

1 4 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 Regnier, 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)

1 4 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 antimutator 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).

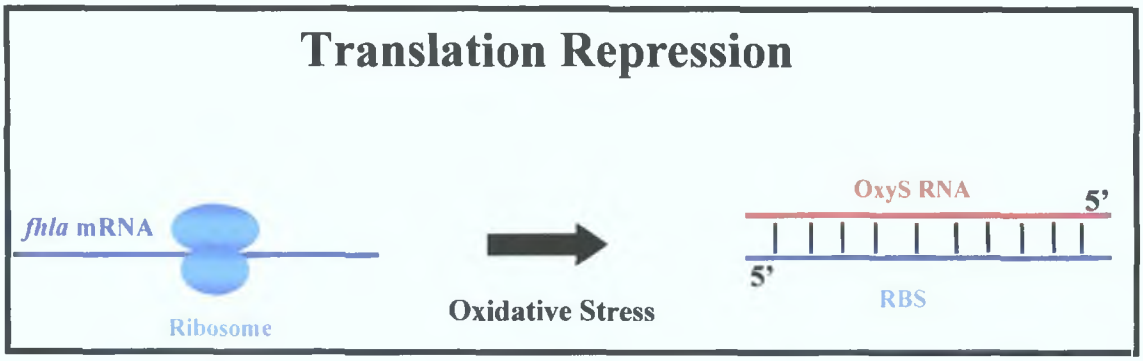


Fig 1.10: Translation repression model with sRNAs

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).

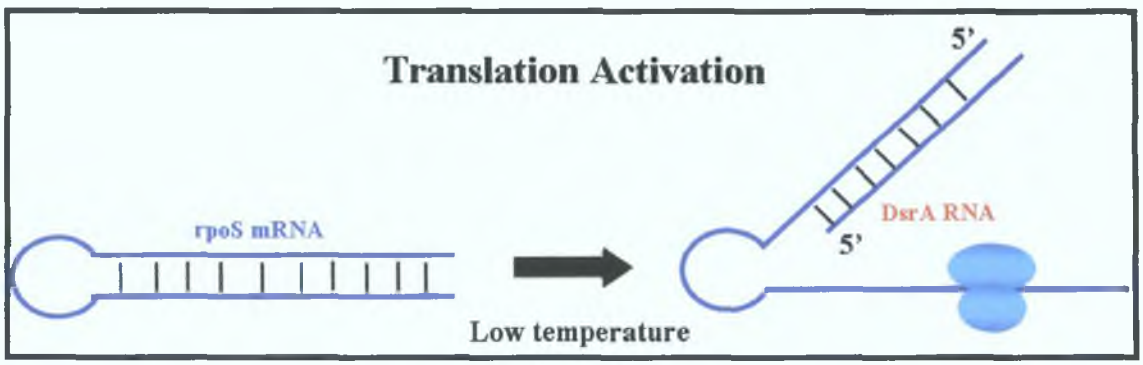


Fig 1.11: Translation activation model with sRNAs

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).

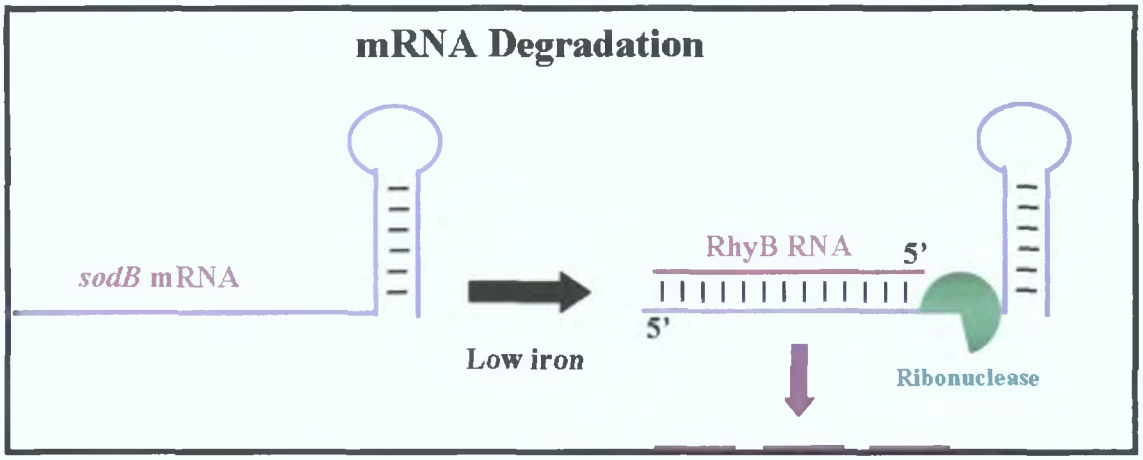


Fig 1.12: *sodB* mRNA degradation model for RyhB sRNAs

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).

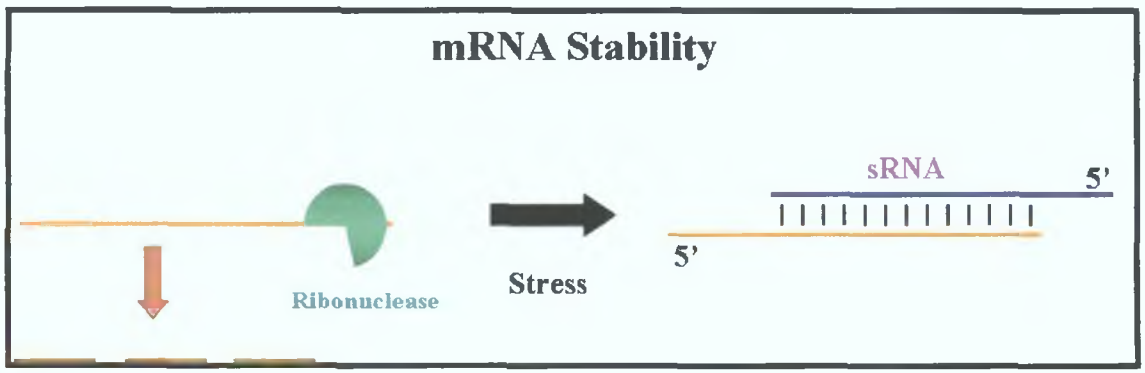


Fig 1.13: mRNA stability model with sRNAs

1 4 3 3 The role of Hfq in iron uptake regulation

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.

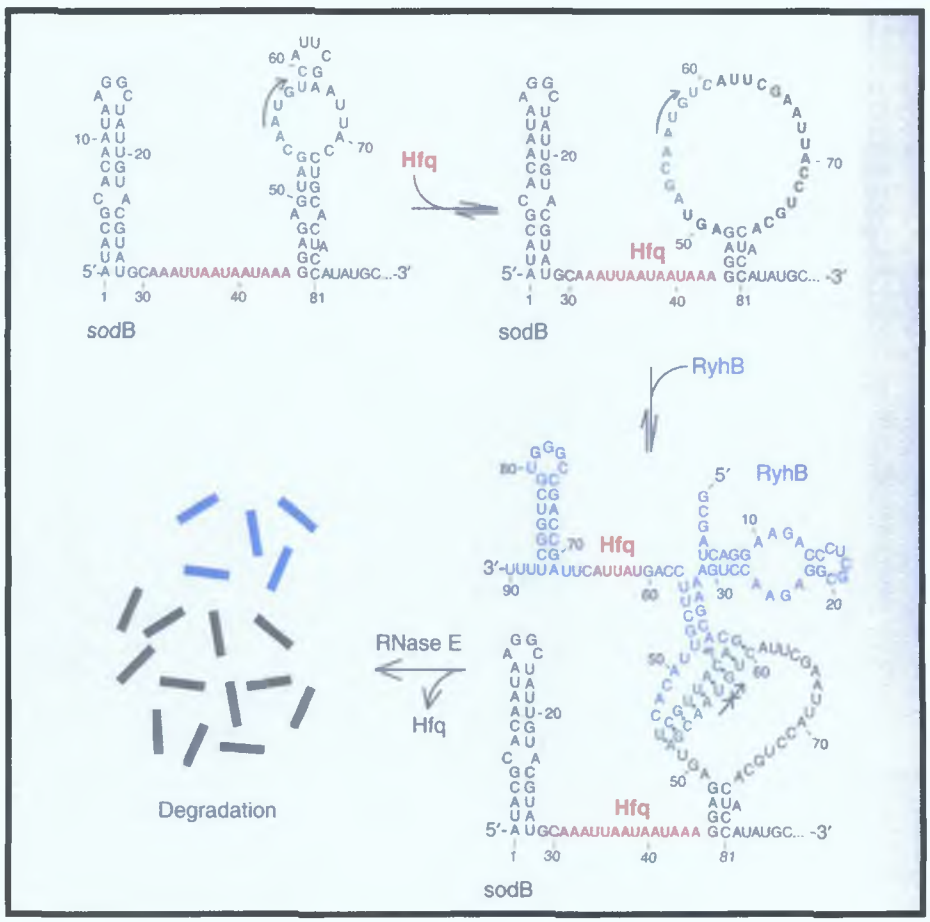


Fig 1.14: Model of sodB mRNA-Hfq-RyhB interaction.
Hfq binds with high affinity to *sodB* mRNA, via an A/U-rich sequence preceding stem- loop **b**. This binding causes the mRNA to adopt a structure in which stem-loop **b**, which follows the Hfq-binding site, is opened out to give a large loop containing 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-Tehrani *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

1 4 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 looses its [4Fe–4S] cluster, thus loosing 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 anguillarum*, 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 N_2-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

Rhizobial strain/species	Host legumes
Sinorhizobium meliloti	Alfalfa
Rhizobium leguminosarum bv. *viciae*	Peas, lentils, vetches
Rhizobium leguminosarum bv. *phaseoli*	Beans
Bradyrhizobium japonicum	Soybeans
Rhizobium japonicum	Soybeans

1 5 2 Infection and nodulation of plants by rhizobia

The infection of legumes by rhizobia is a complex process diagrammatically represented in Fig 1 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)

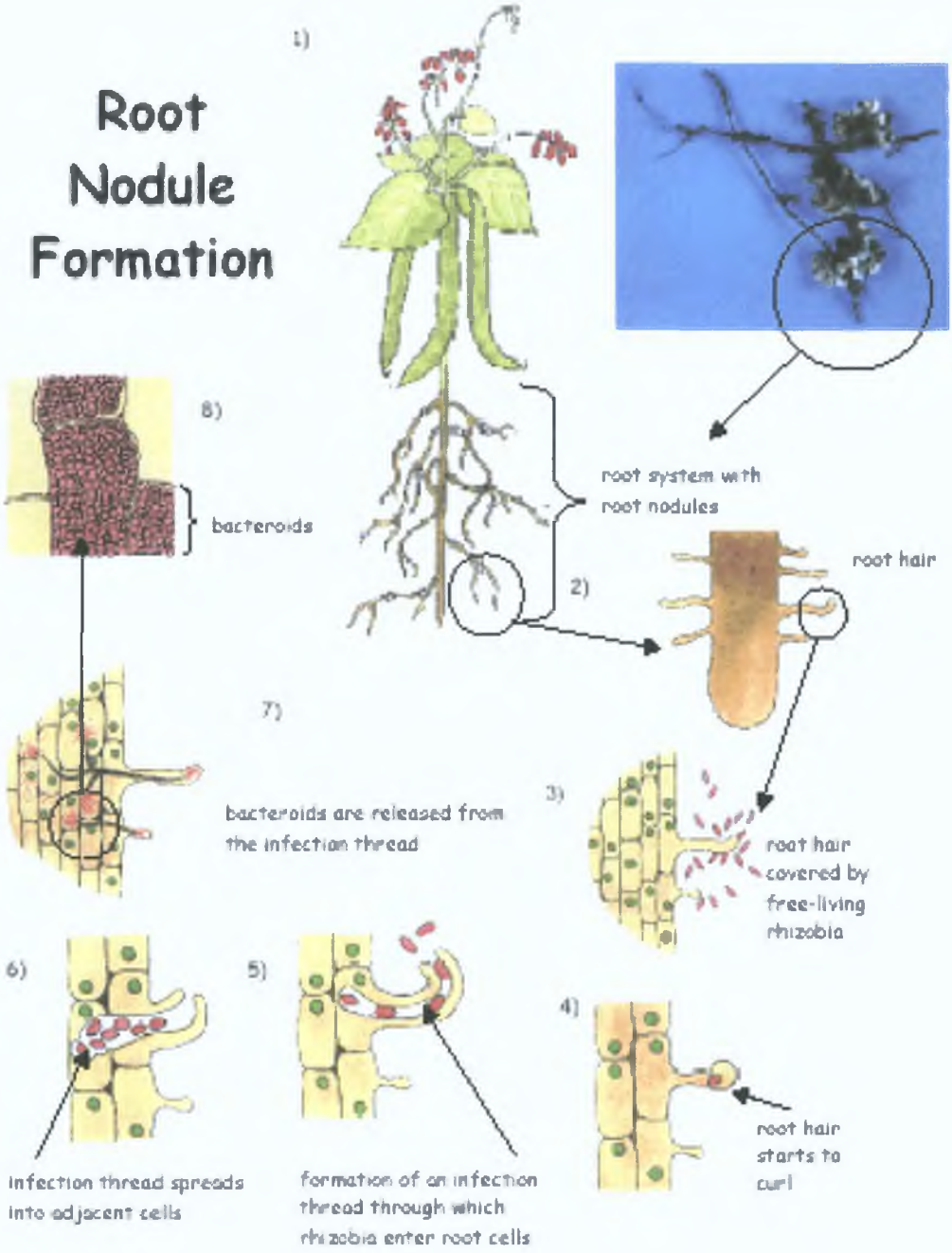


Fig 1.15: Invasion of the plant by rhizobia
(http://www.microbiologyonline.org.uk/forms/rhizobium.pdf)

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_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)

1 6 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

1 6 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

1 6 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-aminolevulinic 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'Brian (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)

1 6 4 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 chracterised

1 6 4 1 The R leguminosarum *siderophore vicibactin*

R leguminosarum biovar *viciae* produces a novel hydroxamate siderophore termed vicibactin (Dilworth *et al*, 1998) Vicibactm 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)

1 6 4 2 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) Schinokinen, 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).

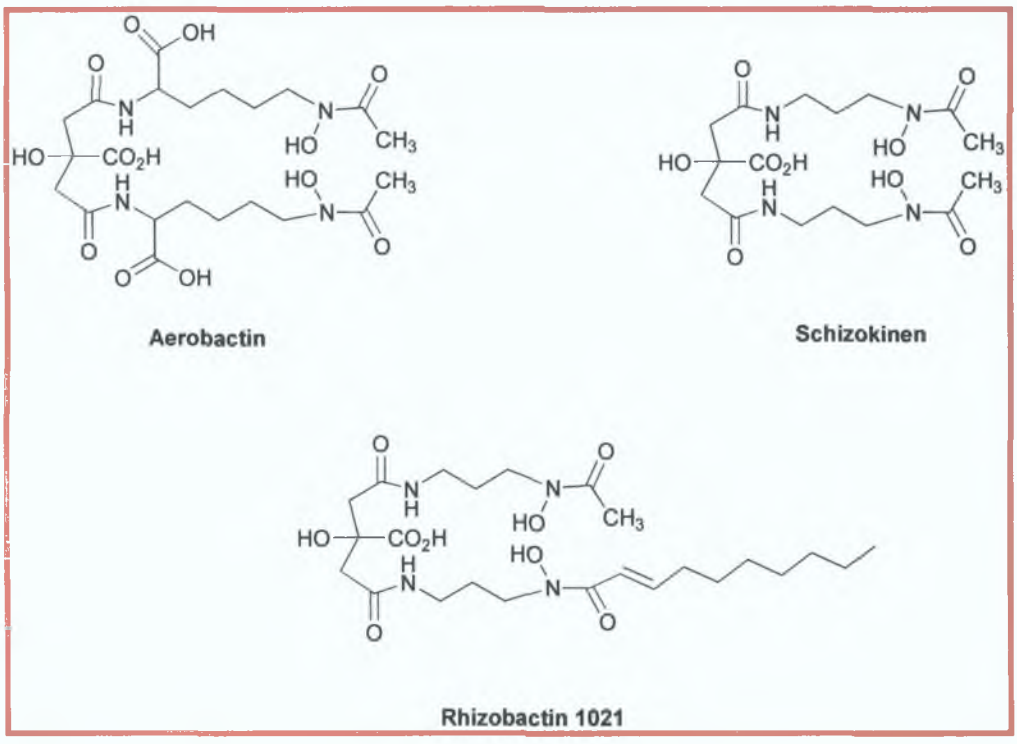


Fig 1.16: Chemical structure of siderophores

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.

1 6 5 Iron Regulation in Rhizobia

1 6 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 legummosarum* (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

1 6 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 legummosarum* (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 Mn^{2+}, 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

1 6 5 3 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 (9×10^5 sites/strand for a genome of 9×10^6 bp), and so cannot be the basis of a binding site for Fur

Friedman *et al* (2003) showed that the affinity of *B japonicum* Fur for its target DNA increases in the presence of the metal DNase I footprinting demonstrated that the binding by *B japonicum* Fur to its binding site within the *irr* promoter is ferrous iron-dependent

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 *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 R leguminosarum*

In *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 *R leguminosarum,* it has been shown that Fur plays a less important role than in most other gram-negative organisms Moreover, Todd *et al* work (2002) have shown that in *R leguminosarum,*the *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 *rirA* transcription is increased two fold under iron replete conditions Also, the *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 *et al*, 2002)

1 6 7 Specific transcriptional regulators in the rhizobia

1 6 7 1 RpoI in R leguminosarum

In *R leguminosarum*, studies revealed that the transcription of genes encoding the siderophore have an absolute requirement for a gene denoted *rpoI*, located upstream from the vicibactin biosynthesis genes (Yeoman *et al*, 1999) From sequence analysis, RpoI 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 *rpoI* itself is up regulated 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 RpoI factor With the exception of *vbsP*, the *vbs* genes are transcribed at higher levels under iron deplete conditions RpoI 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 *rpoI* is not involved in its transcription

1 6 7 2 Irr in B japomcum

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)

1 6 7 3 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)

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 symbioses 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

Strain	Phenotype/Genotype	Source/Reference
Sinorhizobium meliloti		
2011	Wild type, Nod+ Fix+	Meade *et al.*, 1982
2011*rhbA*62	Tn5*lac* insertion in *rhbA*	Lynch *et al.*, 2001
2011*rhbG*25	G212 *lacZ* mutant with Tn5*lacZ* insertion in *rhbG*	Lynch *et al.*, 2001
2011 Str^R	Spontaneous high level streptomycin resistant derivative	Ó Cuív, PhD Thesis (2003)
2011*rhrA*26	Tn5*lac* insertion in *rhrA*	Lynch *et al.*, 2001
2011*rhtA*45	Tn5*lac* insertion in *rhtA*	Lynch *et al.*, 2001
2011*rhbA*62	Tn5*lac* insertion in *rhbA*	Lynch *et al.*, 2001
2011*rirA2*	Kanamycin insertion in *rirA*	This study
Escherichia coli		
DH5α	F^-, *recA1*, *hsdR*17 (r_{K12}-m_{K12}+), *supE*44, ϕ 80*lac* ZΔM15, Δ(*lacZYA-argF*) U169	Bethesda Research
JM109	*recA1 endA1 gyrA96 thi-1 hsdR17* (r_K-m_{K+}), *supE44, relA1 .(lac⁻ proA*B^+) [F′ *traD36 proAB lacI*^{q}*Z ΔM15*].	Stratagene
XL1-blue	*recA1*, *hsdR*17 (r_{K12}-m_{K12}+), *supE*44, *lac*, [F′ *.proA*$^+$*B*$^+$ *lacI*^{q}, *lacZΔM15* T*n10* (Tet^R)]	Stratagene

Strain	Phenotype/Genotype	Source/Reference
XL10-gold	Tet ^{R} ,Δ*(mcr*A)*183* , Δ *(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac* Hte[F′ *proAB lacI*^{q}*Z ΔM15* T*n10* (Tet^{R}) Amy Cam^{R}]	Stratagene
Rosetta blue	*EndA, hsdR17*(r_{K12}-m_{K12}+), *supE44, thi-1, recA1, gyrA96, relA, lac* [F' proA^{+}B^{+}*lacI*^{q}*ZΔM15*::Tn10(Tet^{R})] pRARE	Novagen
H1681	*Thr, se,r fhuA, lacy, rpsL, galK, hsdR, mcrA, fhuF*::LAMpLacMU fur-31 zbf::Tn10	Braun *et al.* (1990)
INVαF'	F^{-}, *recA1*, *hsdR*17(r_{K12}-m_{K12}+) *supE44*, ϕ80*lac* ZδM15, *Δ(lacZYA-argF)*U169	Invitrogen

Table 2.2: Primers

Primer name	Primer sequence (5'→3')
Gene mutation	
rirA-F	CTCGAG TCG CCG AGG CCC ATT CCT TCT
rirA-R	ACTAGT GAA GTC GGC TGT AAA CGG TAT GCG
KanNcoI-F	CCATGG GAC GTT GTA AAA CGA CGG CCA GTG
KanNcoI-R	CCATGG GGA AAC AGC TAT GAC CAT GAT TAC G
Fur-F	ACC ATT CCC CCG GTT ACG CTG ATC
Fur-R	CGT CGG CCT CGC TCA AGG AGT C
KanBss-F	GCGCGC GAC GTT GTA AAA CGA CGG CCA GTG
KanBss-R	GCGCGC GGA AAC AGC TAT GAC CAT GAT TAC G
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 GGC
F-EcoRIpOT1	CG GAATTC ATT ATT TGT AGA GCT CAT CC
R-BglIIR2	GA AGATCT CTC ATT AAG ACA GTA GCG AAC GC
R-BglIIR1R2	GA AGATCT GCA TTT TCG AGA GAG GCG ATA GG
F-BglIIR2	GA AGATCT TCA CAT CCA AGC CGT TCA CCG C
F-BglII+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

Primer name	Primer sequence (5'→3')
MSAfhuF-F	CGGGATCC CGG AAC GAT AGG CCA TAA TCG GG
MSAfhuF-R	CGGGATCC TCC CCA GCC ACT GCC CAG CG
Protein Cloning	
RhrA60-F	CCATGG AGACAATCCGACCG
RhrA60-R	GGATCCAGCGGCGGCTGCCAG
Fur60-F	CCATGG AGAGCCAGAGCAAGAATCGGATCG
Fur60-R	GGATCC GTC CTTGCGCTTCCGGCAATAG
Real-time RT-PCR	
RhbA-F	ATG CCG GCC GAT TTA GCC
RhbA-R:	TCG CGT CTT TCC TGT CGG
RhtA-F	CTATGGAATTGGCAACTACTC
RhtA-R	CGATGATCTCAACGGCAAGC
RhrA-F	TGC CAG CGA CAG GGA AAC G
RhrA-R:	ATG GAG ACA ATC CGA CCG
dppA1-F:	CAC TAC TCT CTT GGC AGC G
dppA1-R	ACG GCT GTA AAC GGT ATG CG
rirA-F:	GCG TCT GAC GAA GCA AAC C
rirA-R	GCG TCT GAC GAA GCA AAC
16S rRNA-F:	ACT TGA GAG TTT GAT CCT GGC
16S rRNA-F:	TCT TTC CCC CGA AGG GCT C
npt-F:	CGC AGG TTC TCCGGC CGC
npt-R:	CTG CGC AAG GAA CGC CCG
Smc02726-F:	ATGCTCAACCGGCATCATCGCCTGGC
Smc02726-R:	CGCGACGATCTTCTTCAGCACGGTCG

Table 2.3: Plasmids

Plasmid	Description	Source/Reference
pOT1	Wide-host-range *gfp* promoter-probe plasmid, Gm^R	Allaway *et al.*, (2001)
pMP220	Wide-host-range l*acZ* promoter-probe plasmid; Km_R	Spaink *et al.*, (1987)
pCR2.1	PCR Cloning Vector: Amp^R, Km^R, *lacZα*	Invitrogen
pUC4K	Amp^R, Source of Km^R cassette	Amersham Pharmacia
pBR322	Tec^R, Source of Tec^Rcassette	Roche
pJQ200sk+	Gm^R, sacB, mob	Quandt *et al.*, (1993)
pRK600	Cm^R, pRK2013 Nm::Tn9, provides transfer functions	Finan *et al.*, 1986
pSTBlue-1	Cloning Vector: Amp^R, Km^R, *lacZα*	Novagen
pQE60	High copy number expression vector	QIgen
pRARE	Cm^R (ArgU, arg W, ile X, glyT, leuW, proL) to improve overexpression yield	Novagen
	pCR2.1 Derived vectors	
pTAFur	500 bp *NcoI/BamHI* product encoding Smc02510 for overexpression	This study
pTARhrA	1 Kb bp *NcoI/BamHI*l product encoding RhrA for overexpression	This study
pTAKanNcoI	Km^R cassette as an *NcoI* fragment	This study
pTAFurM	2.2 Kb bp *BamHI/NotI* fragment encoding for Smc02510 for mutagenesis	This study
pTARirAM	2.2 Kb bp *XhoI/SpeI* fragment encoding for RirA for mutagenesis	This study

Plasmid	Description	Source/Reference
	PSTblue-1Derived vectors	
pSTfur	2.2 Kb bp *BamHI/NotI* fragment encoding for Smc02510 for mutagenesis	This study
pSTfurTec	Tec gene inserted into the *BssHII* site of *smc02510* in pSTfur	This study
	pJQ200ks+ Derived vectors	
pJQrirA	2.2 Kb bp *XhoI/SpeI* fragment in pJQ200ks+ encoding for RirA for mutagenesis	This study
pJQrirAK	Kanamycin cassette in the *NcoI* site of *rirA* in pJQrirA	This study
pJQnrfA	2.2 Kb bp *SpeI/NotI* fragment in pJQ200ks+ encoding for NrfA for mutagenesis	This study
pJQnrfAK	Kanamycin cassette in the *BssHII* site of *rirA* in pJQnrfA	This study
pJQFurTc	3.6 Kb bp *SpeI/NotI* fragment in pJQ200ks+ encoding for Smc02510 with a tetracycline cassette into the BssHII site of the gene for mutagenesis	This study
	pOT1 Derived vectors	
pWT	*HindIII/PstI* promoter region of *rhtX* in pOT1	This study

Plasmid	Description	Source/Reference
pM1	*HindIII/PstI* promoter region of *rhtX* in pOT1 without first repeat	This study
pEN2	*HindIII/PstI* promoter region of *rhtX* in pOT1 without sec repeat	This study
pEN3	*HindIII/PstI* promoter region of *rhtX* in pOT1 without both repeats	This study
pEN4	*HindIII/PstI* promoter region of *rhtX* in pOT1 with extended intergenic region between the repeats	This study
	pQE60 Derived vectors	
pRhrA60	*NcoI/BamHI* fragment encoding RhrA cloned into pQE60 for overexpression	This study
pFur60	*NcoI/BamHI* fragment encoding Smc02510 cloned into pQE60 for overexpression	This study

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
$CaCl_2$ $2H_2O$	0 7 g

Adjusted to pH7 0 with NaOH and volume brought to 1 l with dH_2O 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_2O The solution was then sterilised by autoclaving

♦ Psi broth

After making LB and autoclaving as described above, $MgSO_4$ and KCl were added to final concentrations of 4 mM and 10 mM respectively

♦ SOB Medium

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
KCl	2 5 mM
d H_2O	1 l
pH	7 0

After autoclaving, the solution was allowed to cool to 55°C and sterile solutions of $MgCl_2$ (1M) and Mg_2SO_4 (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*

Agar No 3	7 5 g
dH_2O	550 ml

Following autoclaving solutions of K_2HPO_4, Mg SO_4 and NaCl were added to a final concentration of 0 2 % also added was $CaHPO_4$ to a final concentration of 0 1 % and

$FeCl_3$ 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 dH_2O 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

Tris-HCl	10 mM
Na_2-EDTA	1 mM
pH 8 0	

♦ TES Buffer

Tris-HCl	10 mM
Na_2-EDTA	1 mM
NaCl	50 mM
pH 8 0	

♦ STET Buffer (Holmes and Quigley, 1981)

Tris-HCl	50 mM (5 ml of a 1M solution)
Na_2-EDTA	50 mM (10 ml of 0 5 M solution)
Triton X-100	5 % (v/v)
Sucrose	8 % (w/v)
dH_2O	to 100 ml
pH 8 0	

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

Solution 1

Glucose	1ml (0 5 M solution)
Tris-HCl	0 25 ml (of a 1M solution)
Na_2-EDTA	1 ml (of 0 1 M solution)
dH_2O	to 10 ml

Solution 2

NaOH	2 ml (of 1 M solution)
SDS	1 ml (of 10 % solution)
dH_2O	to 10 ml

Made up every month and stored at room temperature

Solution 3

Potassium acetate	3 M
pH	4 8

To 60 ml of 5 M potassium acetate, 11 5 ml of glacial acetic acid and 28 5 ml of dH_2O was added The resulting solution was 3 M with respect to potassium and 5 M with respect to acetate

♦ 50X Tris acetate (TAE) Buffer

EDTA	100 ml (of 0 5 M solution)
Glacial acetic acid	57 1 ml
Tris	242 g
dH_2O	to 1 l
pH	8 0

Diluted to 1X with dH_2O before use

♦ 6X Gel Loading dye

Bromophenol Blue	0 25 %
Xylene Cyanol	0 25 %
Ficoll (Type 400)	15 %

Made in dH_2O and stored at room temperature following autoclaving

♦ Solutions for Competent Cells

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

Pipes	10 mM
$CaCl_2$	15 mM
KCl	250 mM
pH with KOH	6 7

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

NaCl	175 83 g
Trisodium citrate	88 2 g
pH	7 0
dH_2O	to 1 l

Denaturing solution

NaCl	87 66 g
NaOH	20 g
dH_2O	to 1 l

Neutralising solution

NaCl	87 66 g
Tris	121 1 g
pH	8 0
dH_2O	to 1 l

Washing Buffer

Maleic Acid	11 61g
NaCl	8 76 g
Tween 20	0 3% (v/v)
pH	7 5 (with solid NaOH)
dH_2O	to 1 l

Maleic Acid Buffer

Maleic Acid	11 61 g
NaCl	8 76 g
pH	7 5 (with solid NaOH)
dH_2O	to 1 l

Detection Buffer

Tris	12 11 g
NaCl	5 84 g
pH	9 5
dH_2O	to 1 l

Denhardt's solution (50X)

Ficoll (Type 400)	5 g
Polyvinylpyrrolidone	5 g
BSA (Pentax Fraction V)	5 g
dH_2O	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

SSC	6X
SDS	0 5 % (w/v)
Denhart's solution	5X
Salmon Sperm (10 mg/ml)	1 ml

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

	10%	12%	15%
dH_2O	4 1 ml	3 4 ml	2 4 ml
1.5 M Tris-HCl, pH 8 8	2 5 ml	2 5 ml	2 5 ml
20 % (w/v) SDS	0 05 ml	0 05 ml	0 05 ml
Acrylamide/Bis-acrylamide (30 % / 0 8 % w/v)	3 3 ml	4 0 ml	5 0 ml
10 % (w/v) ammonium persulfate	0 05 ml	0 05 ml	0 05 ml
TEMED	0 005 ml	0 005 ml	0 005 ml
Total	10 005 ml	10 005 ml	10 005 ml

Stacking gel

dH_2O	3 075 ml
0 5 M Tris-HCl, pH 6 8	1 25 ml
20 % (w/v) SDS	0 025 ml
Acrylamide/Bis-acrylamide (30 % /0 8 % w/v)	0 67 ml
10 % (w/v) ammonium persulfate	0 025 ml
TEMED	0 005 ml
Total	5 05 ml

The ammonium persulfate must be prepared on the day

5 X Running Buffer

Tris Base	15 g
Glycine	72 g
SDS	5 g
pH 8 3	

Add dH_2O to 1 l

Sample Buffer

dH_2O	4 0 ml
0 5 M Tris-HCl	1 0 ml
Glycerol	0 8 ml
10 % SDS	1 6 ml
β-mercaptoethanol	0 4 ml
0 05 % (w/v) Bromophenol blue	0 2 ml

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

Staining solution

Acetic acid	100 ml
dH_2O	450 ml
Methanol	450 ml
Bromophenol blue	2 5 g

Destaining solution

Acetic acid	10 ml
dH_2O	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_2PO_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_2PO_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)

100 mM NaH_2PO_4	13 8 g
10 mM Tris Cl	1 2 g
8 M urea	480 5 g

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)

50 mM NaH_2PO_4	6 90 g
300 mM NaCl	17 54 g
10 mM imidazole	0 68 g

Adjust pH to 8 0 using NaOH

Wash buffer (1 l)

50 mM NaH_2PO_4	6 90 g
300 mM NaCl	17 54 g
150 mM imidazole	1 36 g

Adjust pH to 8 0 using NaOH

Elution buffer (1 l)

50 mM NaH_2PO_4	6 90 g
300 mM NaCl	17 54 g
250 mM imidazole	17 00 g

Adjust pH to 8 0 using NaOH

♦ Solutions for Electrophorectic 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
$MgSO_4$	1 mM
Glycerol	10 %

Add before use

PolydIdC	50 µg/ml
BSA	0 1 mg/ml
$MnSO_4$	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, $MnSO_4$ was added on the day to a concentration of 0 1 mM

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

	Tris-acetate (pH 7 4)	40 mM
	100 mM KCl	200 mM
	EDTA	4 mM
	Glycerol	20 %
Add before use	BSA	50 µg/ml
	DTT	1 mM
	PolydIdC	50 µg/ml

10X TBE Running Buffer for RhrA EMSA

Tris	108 g
Boric Acid	55 g
0 5 M EDTA (pH 8 0)	40 ml

♦ Solution for Miller assay

LacZ buffer

Na_2HPO_4	16 1g
NaH_2PO_4	5 5g
KCl	0 75g
$MgSO_4$	0 246g
Mercaptoethanol	2 7 ml
DH_2O	1 L

♦ 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

$$V_a = \frac{(V_t)(x)}{40}$$

Solution

	4%	5%	8%
Accugel	5 0 ml	6 2 ml	10 0 ml
10x TBE for RhrA EMSA / 10x Fur EMSA Running buffer	5 0 ml	5 0 ml	5 0 ml
dH_2O (Ultrapure)	39 9 ml	38 7 ml	34 9 ml
Total	50 ml	50 ml	50 ml

Then 5 µl of 1 M DTT was added to the gel mixture followed by 50 mg of ammonium persulfate and 15 µl 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 dH_2O 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 dH_2O 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 dH_2O 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 dH_2O 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 The

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 (20mg/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 mm, the supernatant was removed to a fresh centrifuge tube Phenol extraction was carried out by adding 700 µl of chloroform isoamyalcohol (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, this 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 mm 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 dH_2O

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)
- 1µl of enzyme/µg of DNA solution (10 U)
- 10 X buffer to a final concentration of 1X
- dH_2O 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.

Standard PCR reaction Mixture

Template DNA	1 µl
Primers (0 6nm/µl)	1 µl of each
Buffer (10X)	5 µl
dNTP Mix (10 mM)	1 µl
Sterile dH_2O	40 µl
RedTaq DNA polymerase	1 µl
Total	50µl

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).

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:

Fresh PCR product		1 µl
PCR2.1 Vector (25 ng/µl)		2 µl
10 X Ligation Buffer		1 µl
Sterile dH_2O		5 µl
T4 DNA ligase (4.0 Weiss U)	1 µl	
Total volume		10 µl

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

DNA	18 µl
DNTPs (0 5 mM)	1 µl of each
Klenow Buffer (10X)	3 µl
Sterile dH_2O	4 µl
Klenow (0 5U/ µl)	1 µl

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

DNA	15 µl
DNTPs labelling mix	2 µl
Hexanucleotide mix	2 µl
Klenow enzyme	1 µl

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 mm 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 dH_2O The solution was stored in the dark at 4°C A 100 µl aliquot of this stock solution was added to 1 l of dH_2O 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 manufactures instructions Briefly, the gel slice were excised with a sterile scalpel and weighed Three volumes of gel solubilising 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 mm 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 mm 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 Psi 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 100µl 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

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 with 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 removes 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 approximatively 12-24 hrs (See Fig 2 2)

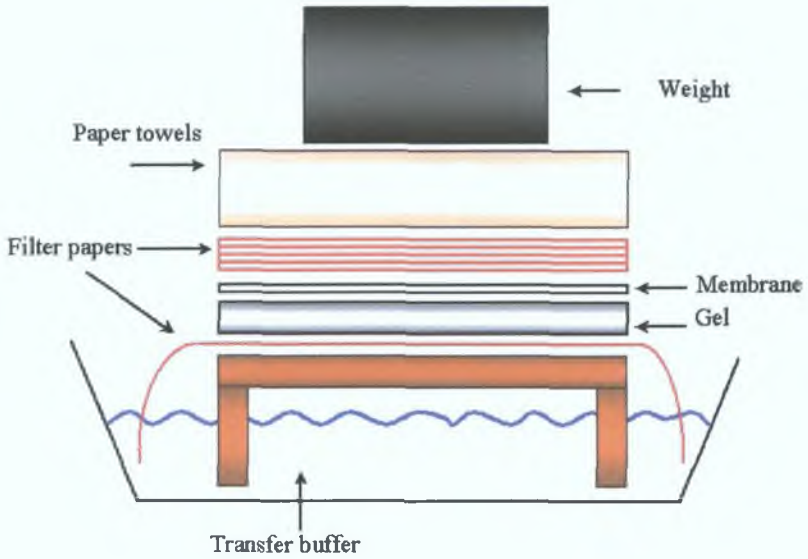


Fig 2.2: Southern Blot transfer of the DNA from the agarose gel to the nitrocellulose membrane.

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 cm^2 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 cm^2 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 mm 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 *lacI*^{q} 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 mm 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 dH_2O 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 dH_2O, 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 mm, 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 *BamHI* 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-^{32}P dATP (4000 Ci/mmol, 10 mCi/ml)
- 20 U T4 polynucleotide kinase
- dH_2O to a final reaction volume of 50 µl

DNA (30 pmoles)	X µl
Buffer (10 X)	1 66 µl
γ-^{32}P dATP	5 00 µl
T4 polynucleotide kinase (5 U/µl)	1 33 µl
H_2O	X µl
Total	16 66 µl

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 EMSA binding reaction

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

Binding Mix	
Binding reaction buffer (5 X)	4 µl
Non-specific competitor DNA poly dI-dC (1µg/µl)	1 µl
BSA (1 mg/ml)	2 µl
Total	7 µl

Binding reaction	
Binding Mix	7 µl
^{32}P-labeled DNA probe (20,000 cpm/µl)	1 µl
Purified protein/Extract	X µl
Tris/HCl pH8 0	X µl
Total	20 µl

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 were first prerun for 20 mm 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

Binding Mix	
Binding reaction buffer (4 X)	5 µl
Non-specific competitor DNA poly dI-dC (1 µg/µl)	2 µl
BSA (1 mg/ml)	2 µl
Total	9 µl
Binding reaction	
Binding Mix	9 µl
^{32}P-labeled DNA probe (20,000 cpm/µl)	1 µl
Proteins Extract	X µl
Sonication buffer	X µl
Total	20 µl

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 mm 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 approximatively 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 H_2O 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 H_2O 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 flurorescent 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 house keeping genes

Because PCR can even detect a single molecule of DNA, RNA samples were digested with Deoxyribonuclease I (DNaseI) 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 DNaseI 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 H_2O	8 µl
10 X Reaction buffer	1µl
Amplification Grade DNase I (1 U/µl)	1µl

The reaction was then incubated for 15 min at room temperature Then, 1µl 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 H_2O 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
dNTP mix (20 mM)	1 µl
$MgCl_2$ (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 H_2O	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, $MgCl_2$, 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 H_2O	8 5 µl
Forward primer (0 4 µM)	1 µl
Reverse primer (0 4 µM)	1 µl

Samples were quantififed using the Rotor Gene™ 3000 multiplex system (Corbett research) under the following thermo-cycling conditions

First cycle (Denature)	95°C for 15 min 1 cycle
Second cycle (Cycling)	95°C for 20 sec 50°C for 30 sec 50 cycles 95°C for 30 sec
Third cycle (hold)	60°C for 1 min 1 cycle
Final cycle (melt)	50-99°C rising by 1°C each step, waiting for 15 sec on first step, then 5 sec for each step afterwards

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 Na_2CO_3 The cells were then pelleted by centrifugation at 13, 000 rpm in a microfuge tube and OD_{420} of the supernatant recorded β-galactosidase activity was calculated using the equation below

$$\text{Miller units} = \frac{OD_{420} \times 1000}{V \times T \times OD_{600}}$$

OD_{420} = the absorbance of the supernatant at 420 nm

OD_{600} = tha 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* 2011*rhrA*26 and 300 µM for *S meliloti* 2011 and 2011*rirA*2) 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 ands 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 supernants 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 *rhtX* (Ó Cuív *et al.*, 2004). Fig 3.1 shows the positions and orientations of the above mentioned genes.

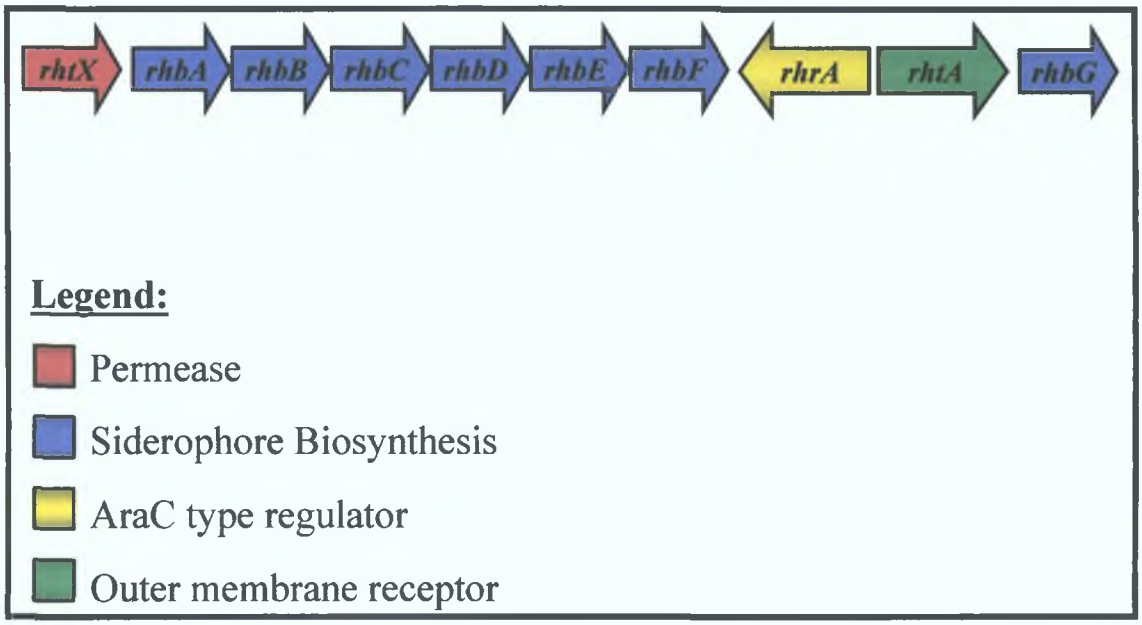


Fig 3.1: Organisation of the rhizobactin 1021 regulon

Fur in *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 *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 *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)

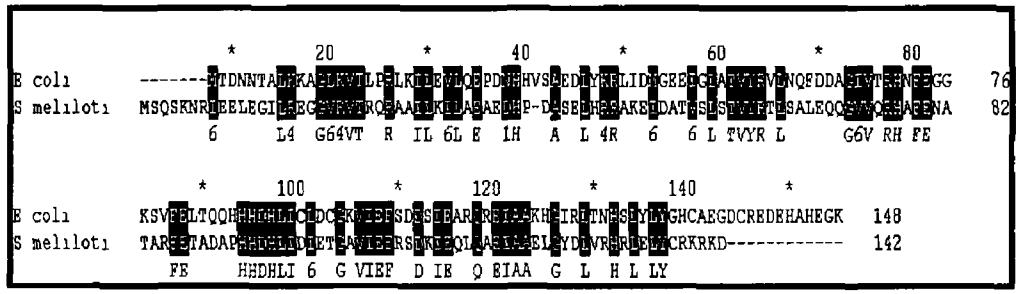


Fig 3 2. Amino acid sequence alignments of Fur from *S meliloti* 2011 (Smc02510) and Fur from *E. coli* K12.

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.

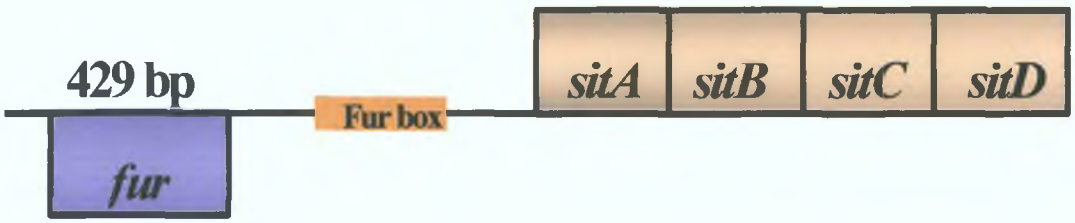


Fig 3.3: Chromosomal location of *fur*

In the intergenic region between *fur* and *sitABCD* a putative 'Fur box' was identified (Fig. 3.4).

Fig. 3.4: Alignment of the putative *S. meliloti* 'Fur Box' to the *E. coli* 'Fur box' consensus sequence.

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.

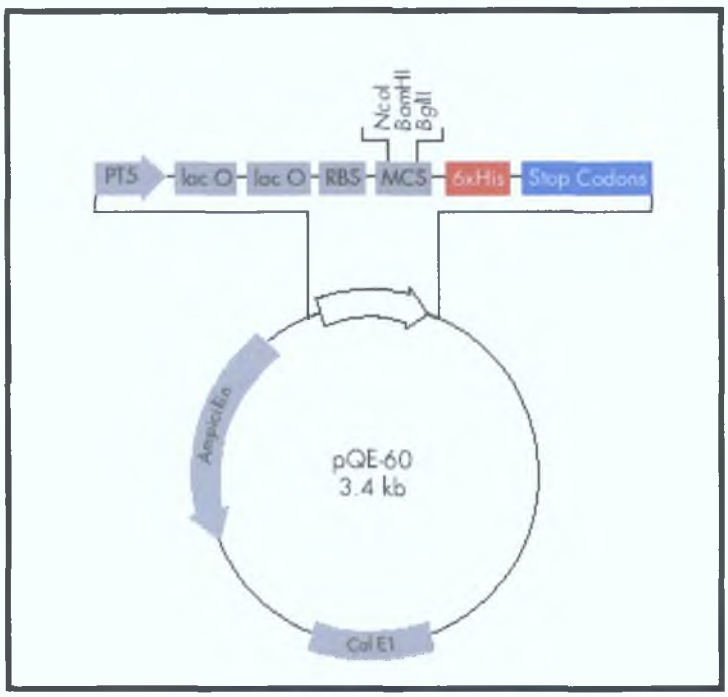


Fig 3.5: pQE60 map (Qiagen)

The *fur* gene from *S. meliloti* 2011 was amplified from *S. meliloti* genomic DNA by PCR. The restriction sites *NcoI* 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 *NcoI* 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 *Nco*I-*BamH*I 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.*

PQE-60

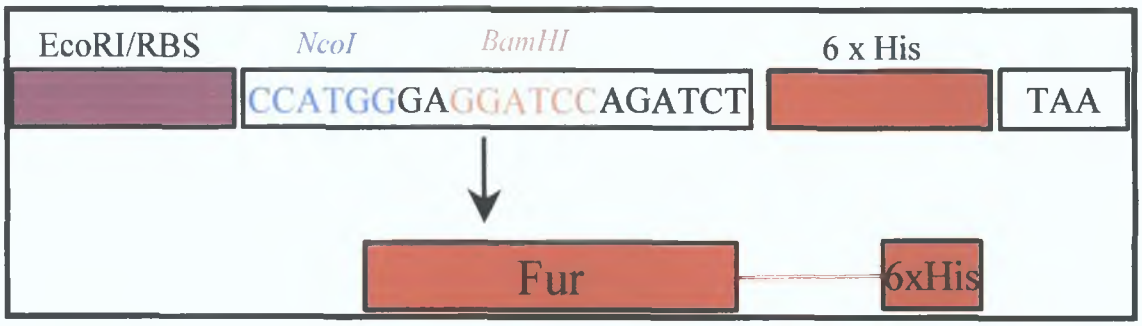


Fig 3.6 : Cloning of the *fur* gene into the multiple cloning site of pQE60

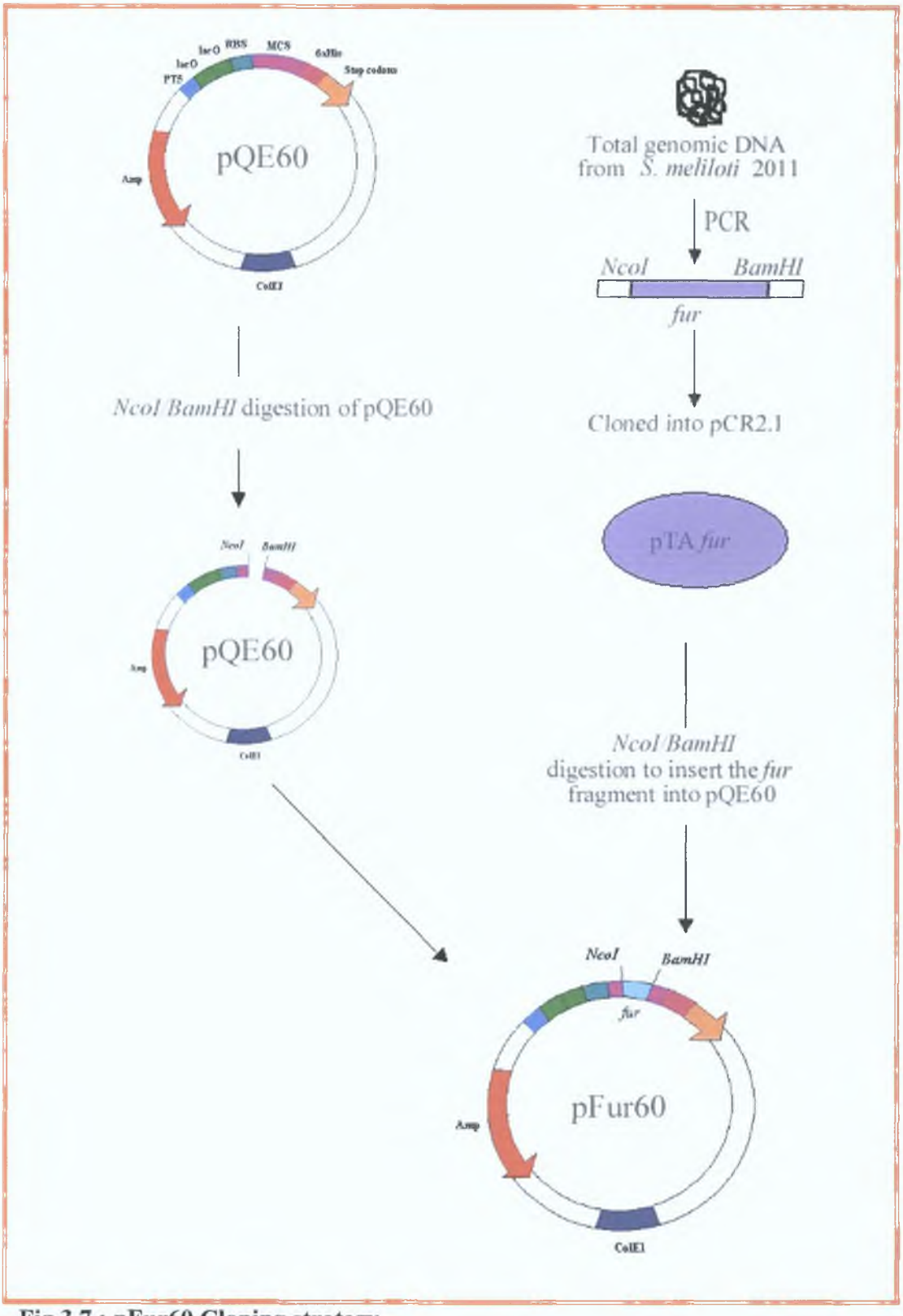


Fig 3.7 : pFur60 Cloning strategy

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.

PCR Conditions
Annealing Temperature 66°C
Annealing Time 1 min
Extension Time 72°C for 1 min

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

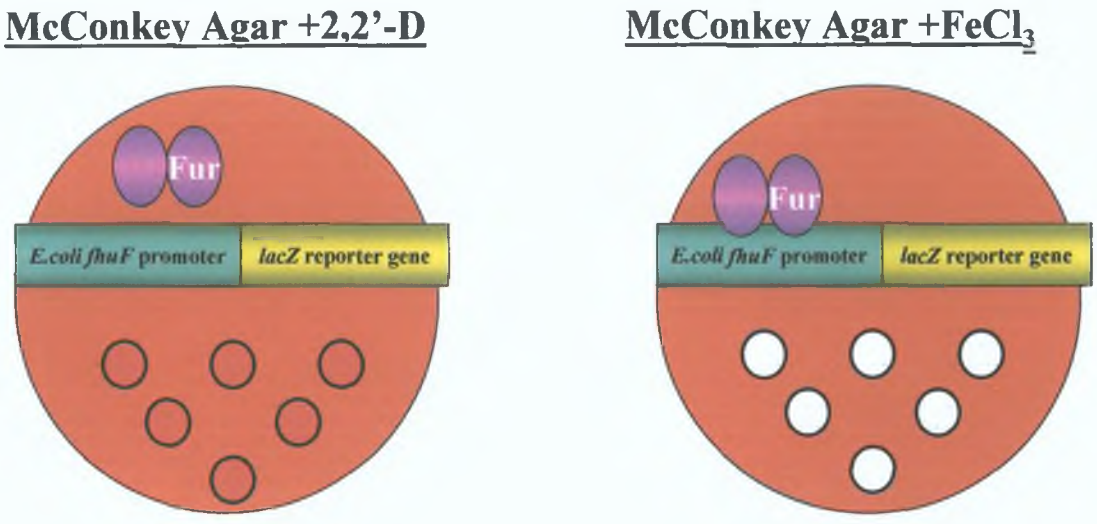


Fig 3.8: Principle of the complementation assay

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 $FeCl_3$ 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

Strain	McConkey Agar + $FeCl_3$	McConkey Agar + 2,2'-dipyridyl
***E. coli* H1681 + pFur60**	**Pink**	**Red**
***E. coli* H1681 + pQE60**	**Red**	**Red**

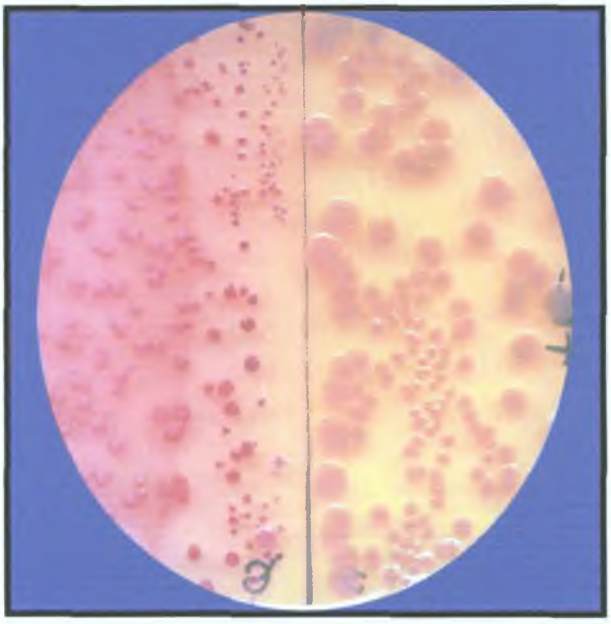


Fig 3.9: Fur complementation on Mc Conkey Agar supplemented with $FeCl_3$ (iron replete conditions).

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.

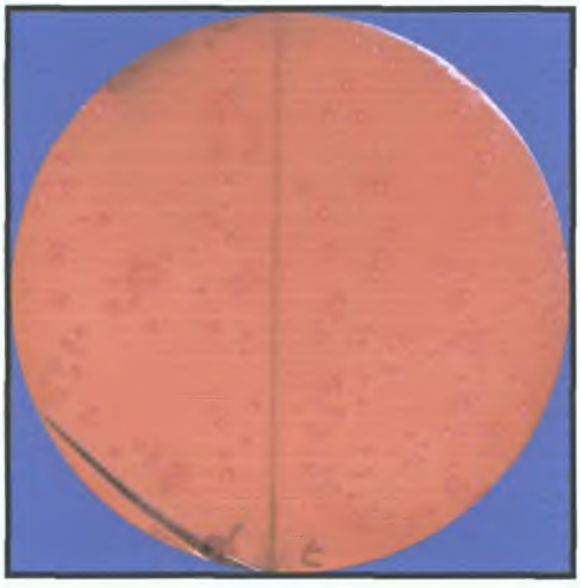


Fig 3.10: Fur complementation on Mc Conkey Agar supplemented with 2,2'-dipyridyl (iron deplete conditions).

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 determinate 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).

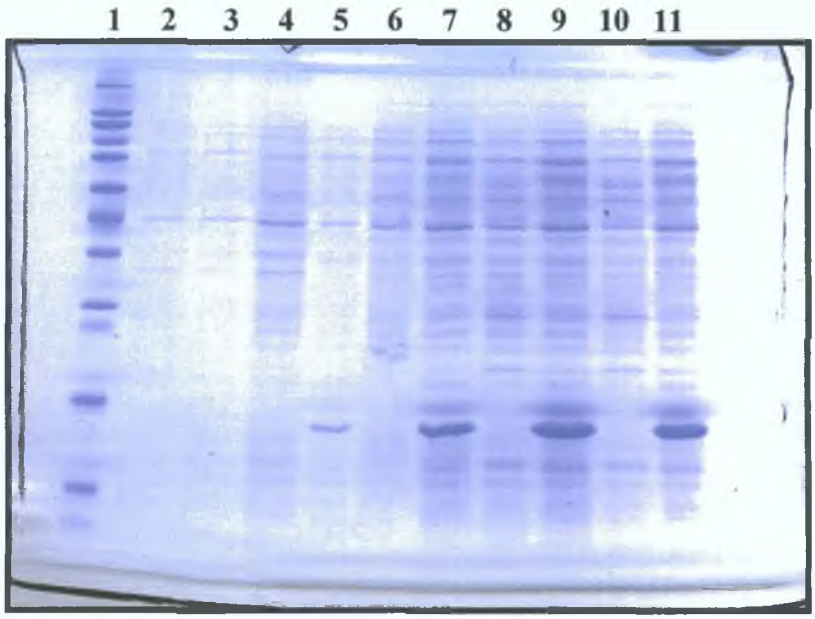


Fig 3.11: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: Time course of expression of Fur under denaturing conditions.

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

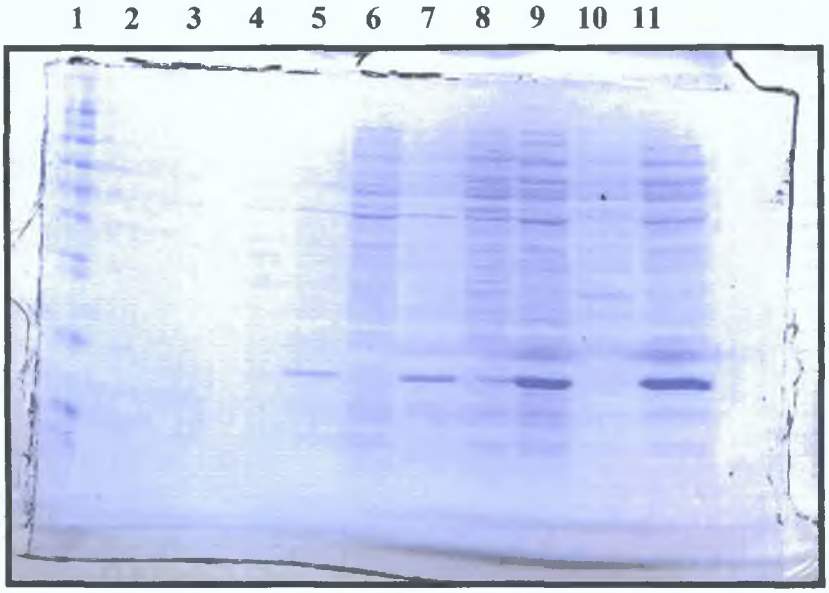


Fig 3.12: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: Time course of expression of Fur under native conditions.

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).

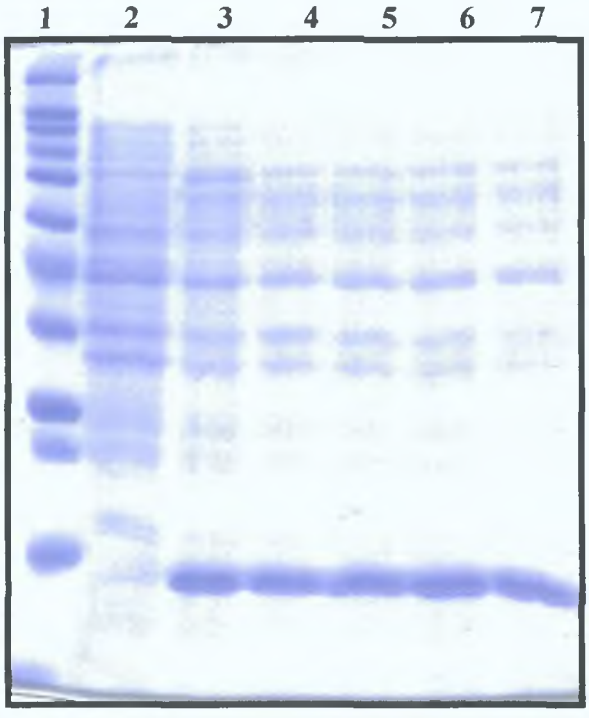


Fig 3.13: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: Optimisation of the concentration of IPTG for mini prep under denaturing conditions.

Lane 1: Ladder

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

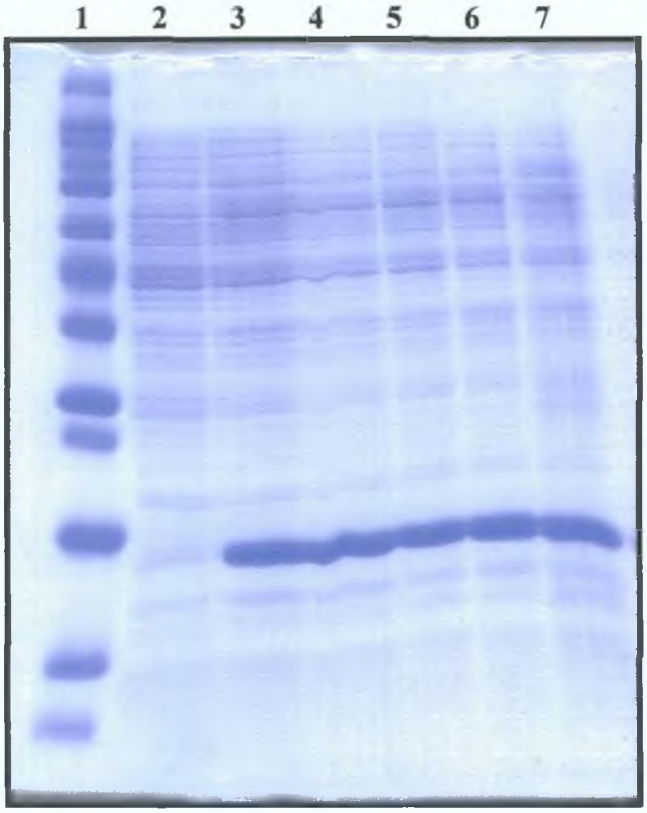


Fig 3.14: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: Optimisation of the concentration of IPTG for mini prep under native conditions.

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 somcator (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)

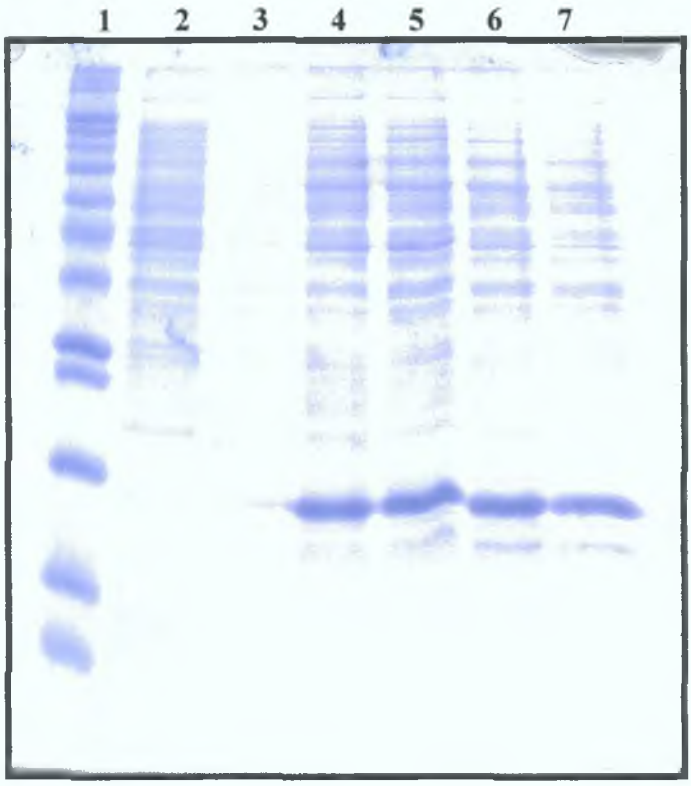


Fig 3.15: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: Optimisation of the time of sonication for mini preps under native conditions. Samples were induced with 0.1 mM IPTG.

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

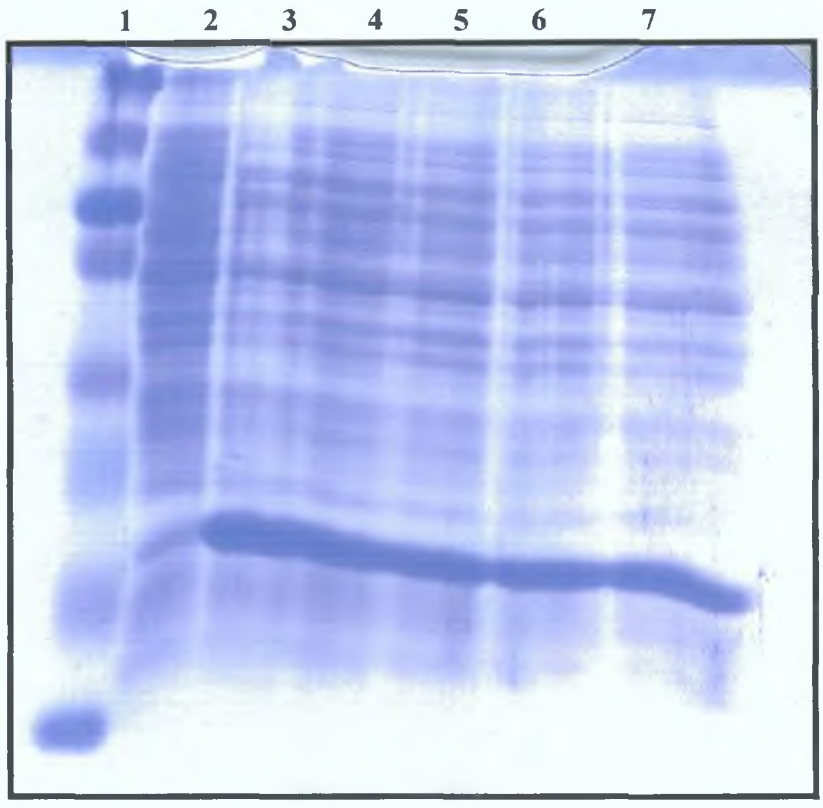


Fig 3.16: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: Optimisation of the time of sonication for mini preps under denaturing conditions.

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).

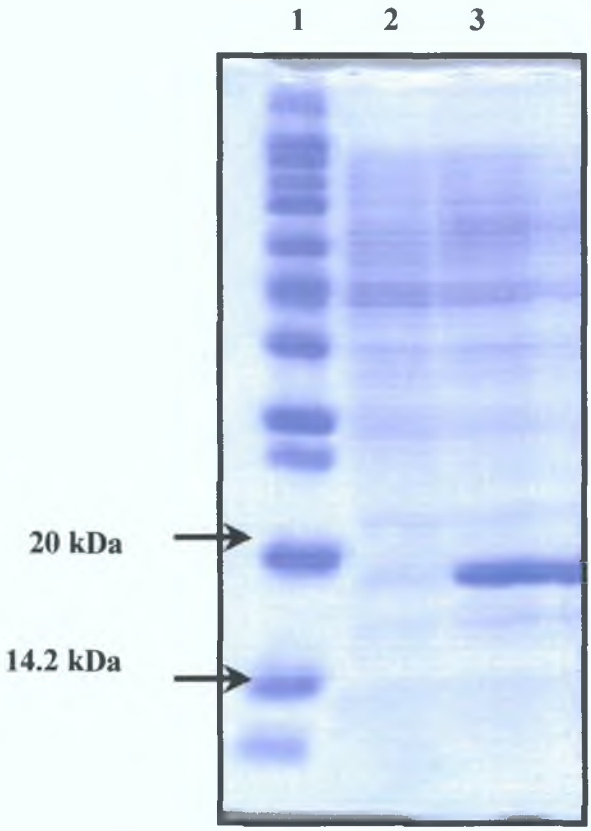


Fig 3.18: SDS-PAGE gel loaded with protein preparations from *E. coli* XL10 Gold, pFUR60: *S. meliloti* recombinant Fur expressed under native conditions

Lane 1: ladder
Lane2: 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)

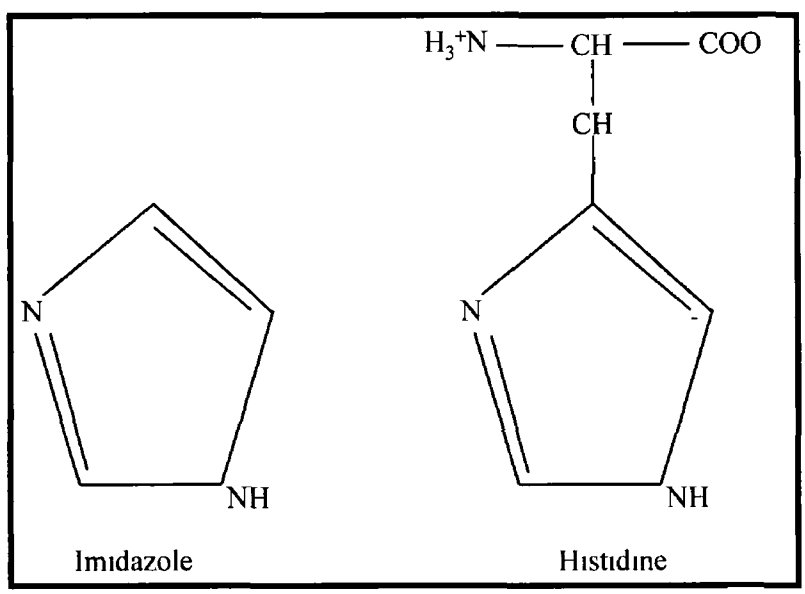


Fig 3 19 Chemical structures of histidine and imidazole

Since the *S meliloti* 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 minimize 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 determinate 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)

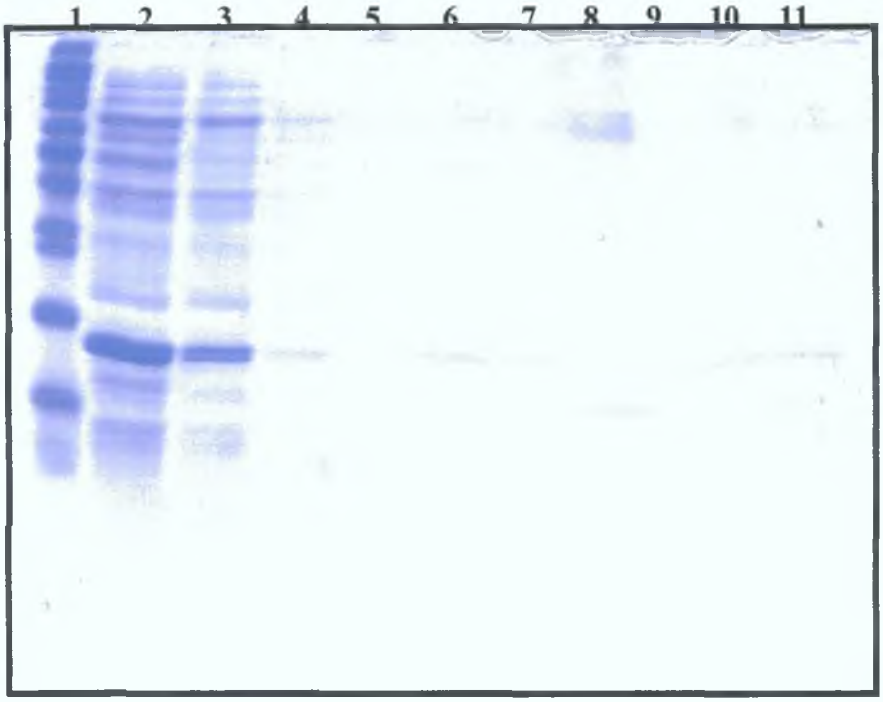


Fig 3.20: 15 % SDS polyacrylamide gel. Native protein preparations from *E. coli* XL10 Gold, pFur60 analysed by addition of washing buffers with a gradient of imidazole concentrations.

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

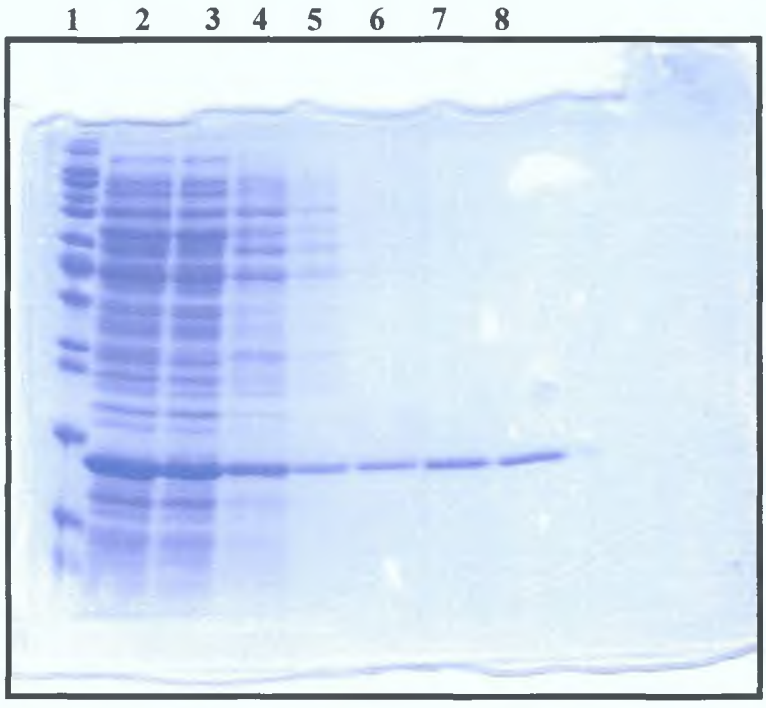


Fig 3.21: 15 % SDS polyacrylamide gel following purification of native Fur protein and IMAC purification of recombinant His-tagged-Fur.

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 1with 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.

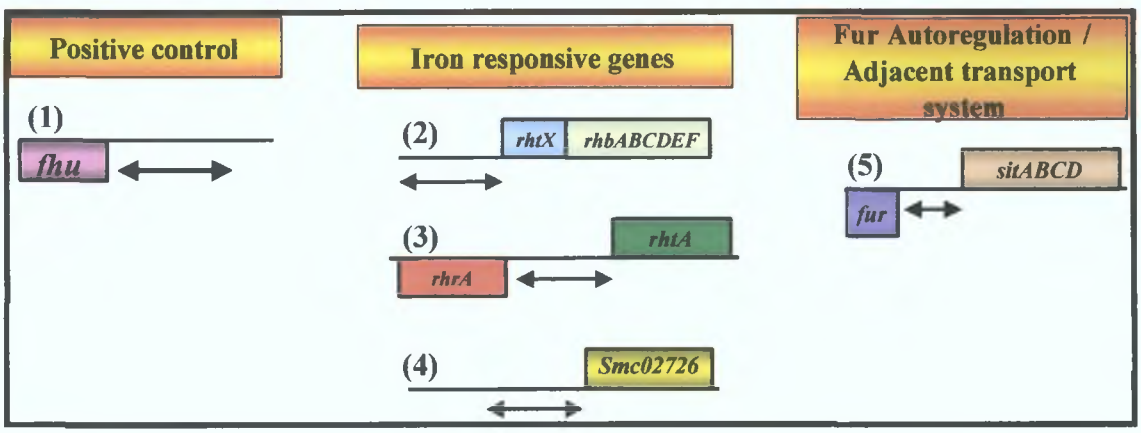


Fig 3.22: DNA probes for mobility shift assay.
The amplified regions are shown (↔) for the *fhuF* promoter region *of E. coli*, a positive control (1), iron responsive promoters from *S. meliloti* (2,3 and 4) and the promoter region of the *fur* gene in *S. meliloti (5)*.

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 *rhtXrhbABCDEF* 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 *rhtA* is known to be iron responsive and therefore the intergenic region between *rhrA* and *rhtA* was also investigated for Fur binding

Also, the promoter region of *smc02726*, a gene encoding the outer membrane receptor for haem utilisation in *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 *E coli*, Fur expression is constitutive However, *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 *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 *fhuF*:

MSAfhuF-F:

CGGGATCC CGG AAC GAT AGG CCA TAA TCG GG

MSAfhuF-R:

CGGGATCC TCC CCA GCC ACT GCC CAG CG

CGGGATCCCGGAACGATAGGCCATAATCGGGATAGTAATCTAAATG
ATAATGATTGCTAATCATAGCGATAGGTTTACCCGATAGCAAGGGAT
TTATCTGGCTTGCAAATGATAAAAATTATCATATGATATTGGTTATCA
TTATCAATGAAAGAGATGAAATCATGTTGCAACGTACGCTGGGCAGT
GGCTGGGGAGGATCCCG

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

CGGGATCCCCTATCGCCTCTCTCGAAAATGCGTTCGCTACTGTCTTA
ATGAGGTTCGCTCACATCCAAGCCGTTCACCGCACGTCCATTTAAAG
ATGACGGCAACACTCATGTTTATCGTCAGACAATGTTGCCGGGCAGT
GGCAGTTTTCGGGATCCCG

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

MSArhra-R:

CGGGATCC T GCC CAT AA CGC CCC CTG CGC

CGGGATCCGTCGTGCGCCAGCCTTTCCTGTTGACGTTCGCATGCGTC
CAAATGAGGTTCGCCATTATCCAAGCGGCGAACACCCTTAGCCCATA
AAACATGACTTAAATAGTCTTGTATTGGCAATTTGCCCGCCCACCGG
CAGCGGCAATTGTTTTCTGGTGCGCAGGGGGCGTTATGGGCAGGAT
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

CGGGATCCGGACCAGTCCTTTGAAAGTGTTGGCCGGGCTTGCTGTT
GAGCGGCGAACTCAAGGGCTGGCTGGGCAGTGCGGAATTGGGCAAG
GCGATCTATTTGCGCCTTATTTTCGTCACATAAGAAAACGGATCCCG

Probe length: 139bp. 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

CGGGATCCCCCGCGACACTAGCCAAGGGGACACCTTTTGGAAATAG
CTAGTTGCAAATGCTTCTCATTTGCATTGACTTATGCAGACCATTCG
CCTACCCATATTATGGTTCGCAAAGAGGAGAGCCGGGGATCCCG

Probe length: 137bp. 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.

PCR Conditions
Annealing Temperature 64°C
Annealing Time 1 min
Extension Time 72°C for 1 min

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).

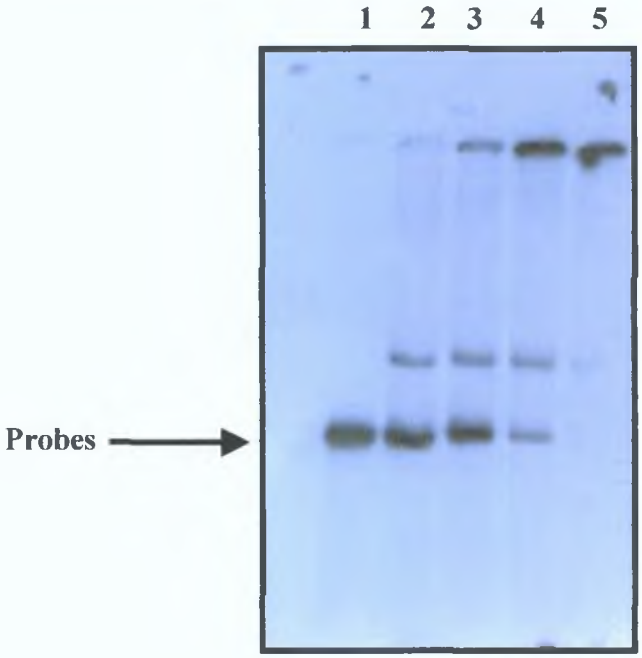


Fig 3.23: EMSA with purified Fur and the promoter region of *fhuF*

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 *rhtXrhbABCDEF* (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).

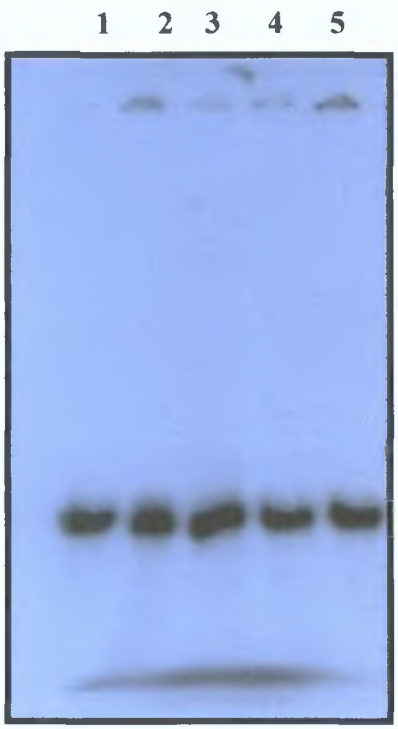


Fig 3.24: EMSA with purified Fur and the region from the heme receptor *smc02726*

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

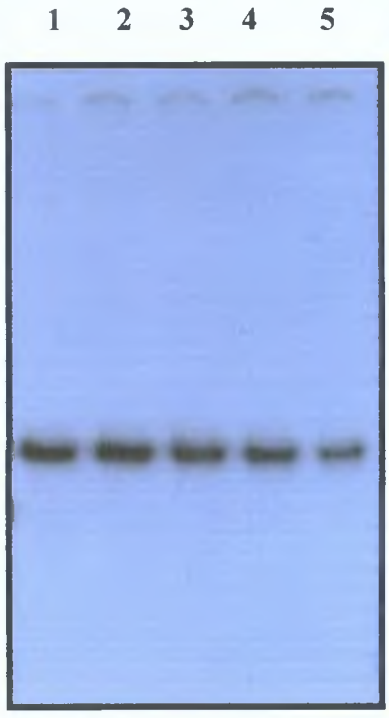


Fig 3.25: EMSA with purified Fur and the intergenic region between *rhrA* and *rhtA*

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 *rhtX*

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

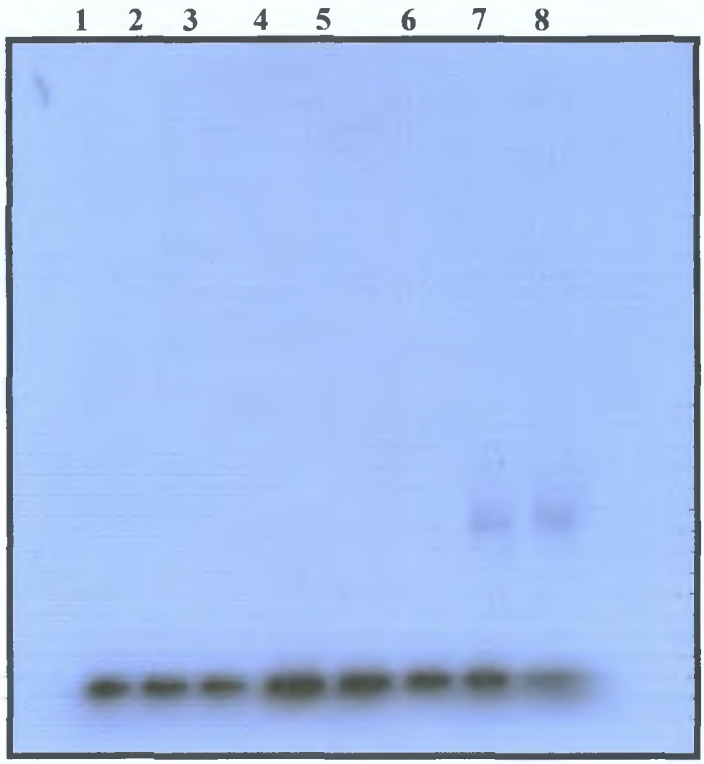


Fig 3.28: EMSA with *E. coli* extracts from cells carrying either pQE60 or pFUR60 and the region upstream *fhuF*

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).

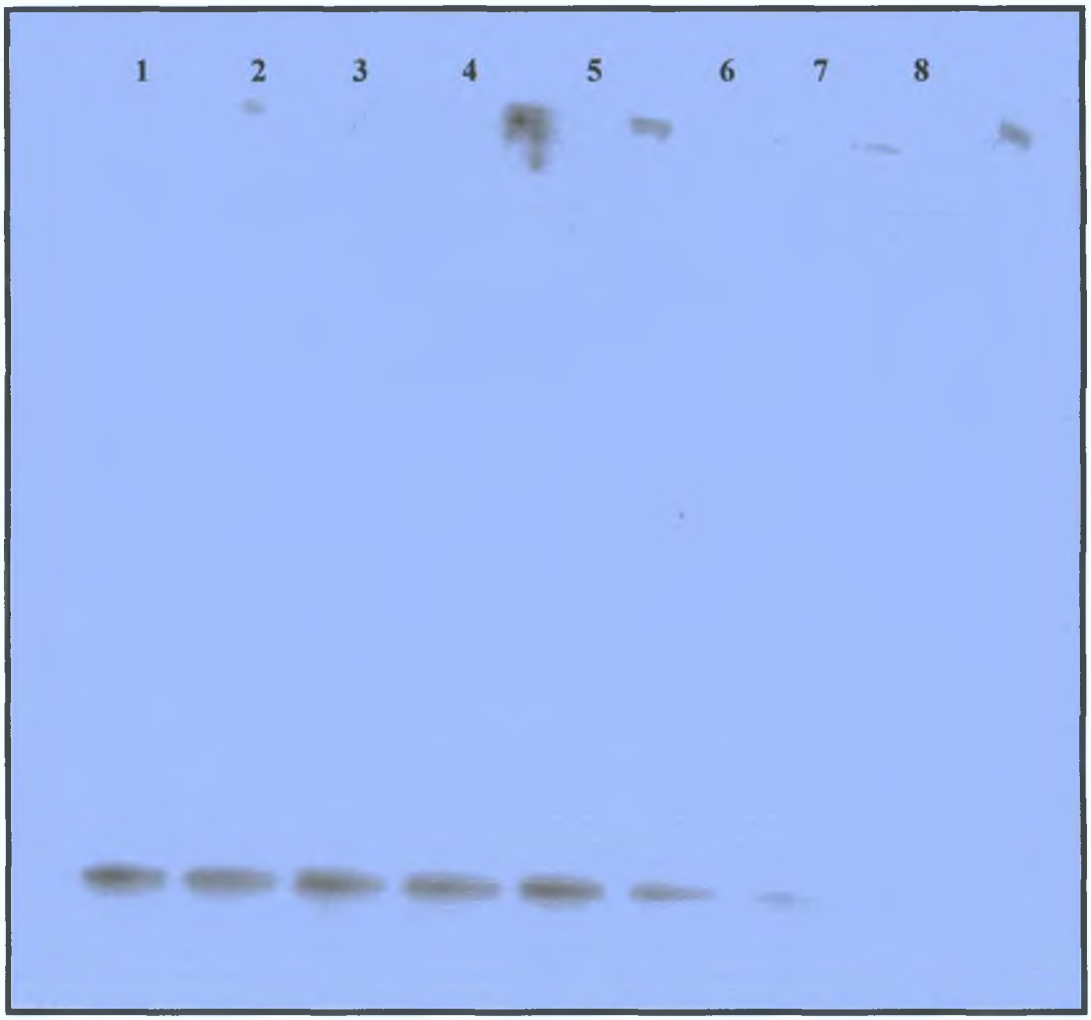


Fig 3.29: EMSA with *E. coli* extracts from cells carrying either pQE60 or pFUR60 and the intergenic region between *fur* and *sitA*

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 characterisation 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 *fhu*F 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 *fhuF* 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 metallorepressor 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 dimetal 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 *at 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 *rirA* and of *rhrA* in *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 *rirA* 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

BlastP results: Hit Description			
rirA		SMc00785_AA (154 aa)	
		CONSERVED HYPOTHETICAL PROTEIN	
Begin position	End position	Begin position	End position
1	432	1	143
Blast score		617	
Expect value		5e-66	
Identity		84%	
Positive		92%	
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 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*

Protein	Organism	Accession	Homology
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.

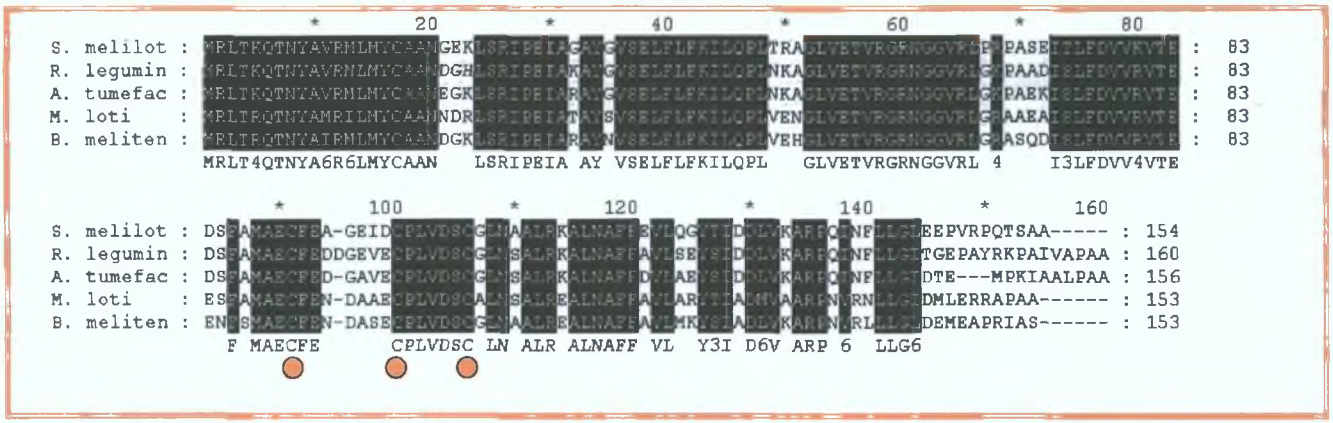


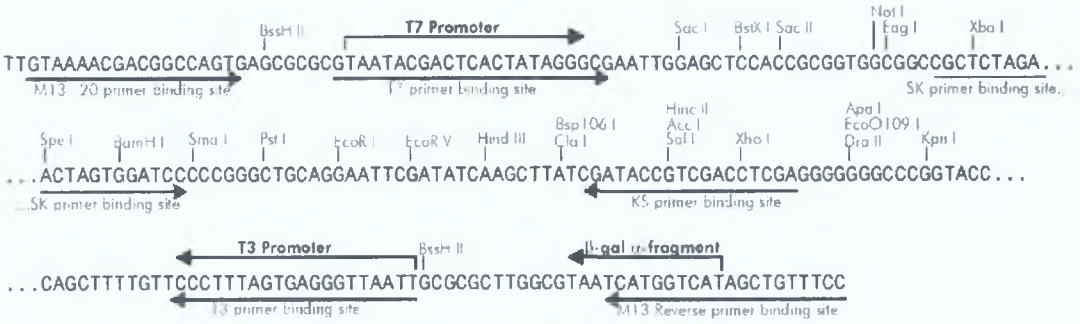
Fig 4.1: Comparison of *S. meliloti* RirA (SMc00785) to other RirA homologues.
The *S. meliloti* RirA protein is aligned with very close homologues from *R. leguminosarum*, A. tumefaciens, *M. loti*, and *B. melitensis*. The orange circle shows the three C-terminal cysteines characteristic of RrF2 proteins.

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 sheme in Fig 4.3.



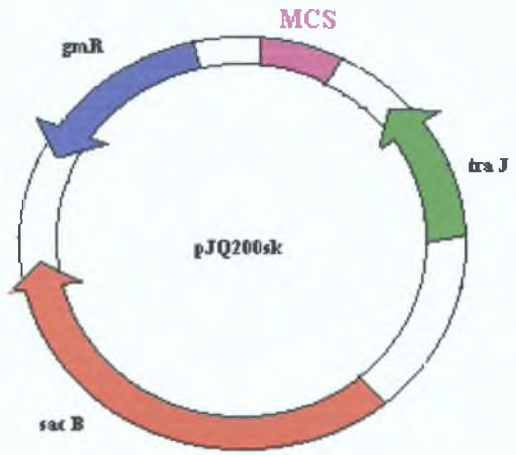


Fig 4.2: Map and Multiple cloning site (MCS) of pJQ200sk (Quandt and Hynes, 1993), gm; gentamicin resistance, tra; transfer.

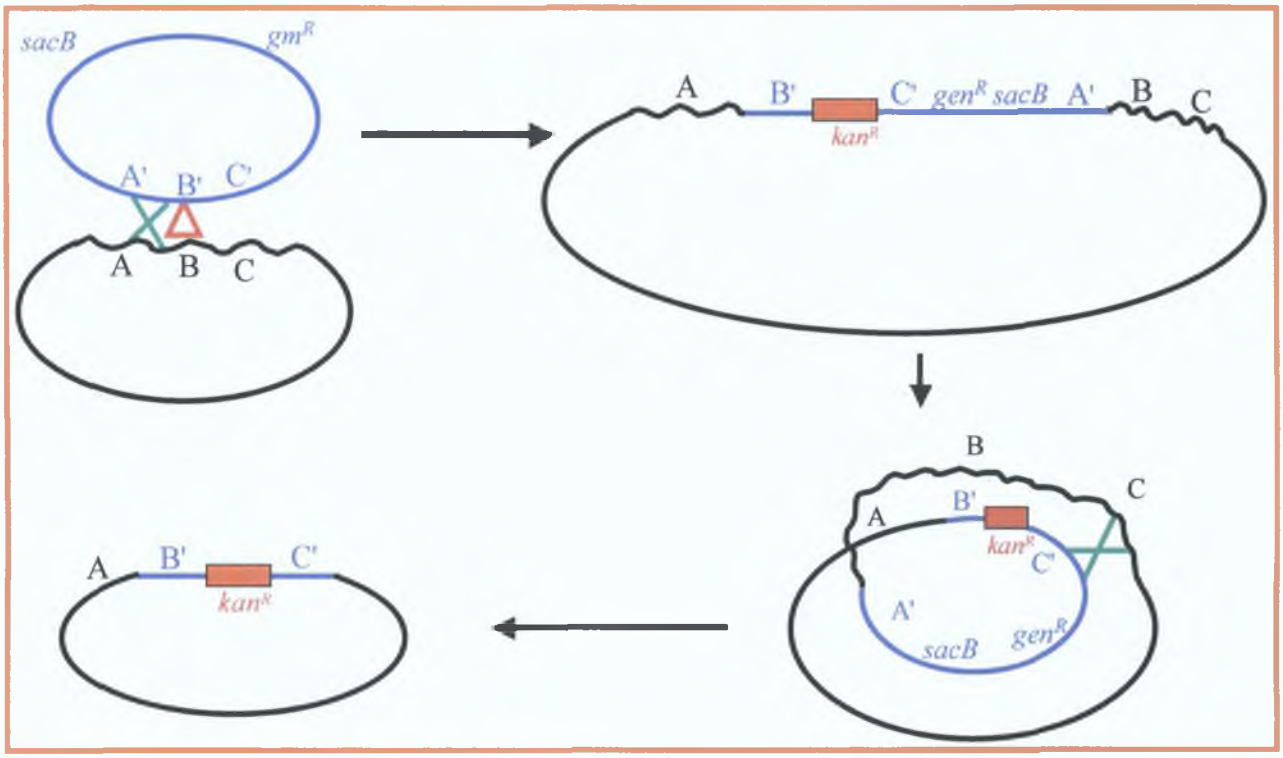


Fig 4.3: Recombination event scheme.
A', B', C' denote the copies of A, B, C cloned into the pJQ200sk vector. The kanamycin resistance cassette is inserted in B' (Δ). The sites of recombination events are indicated (X).

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, SalI, 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.

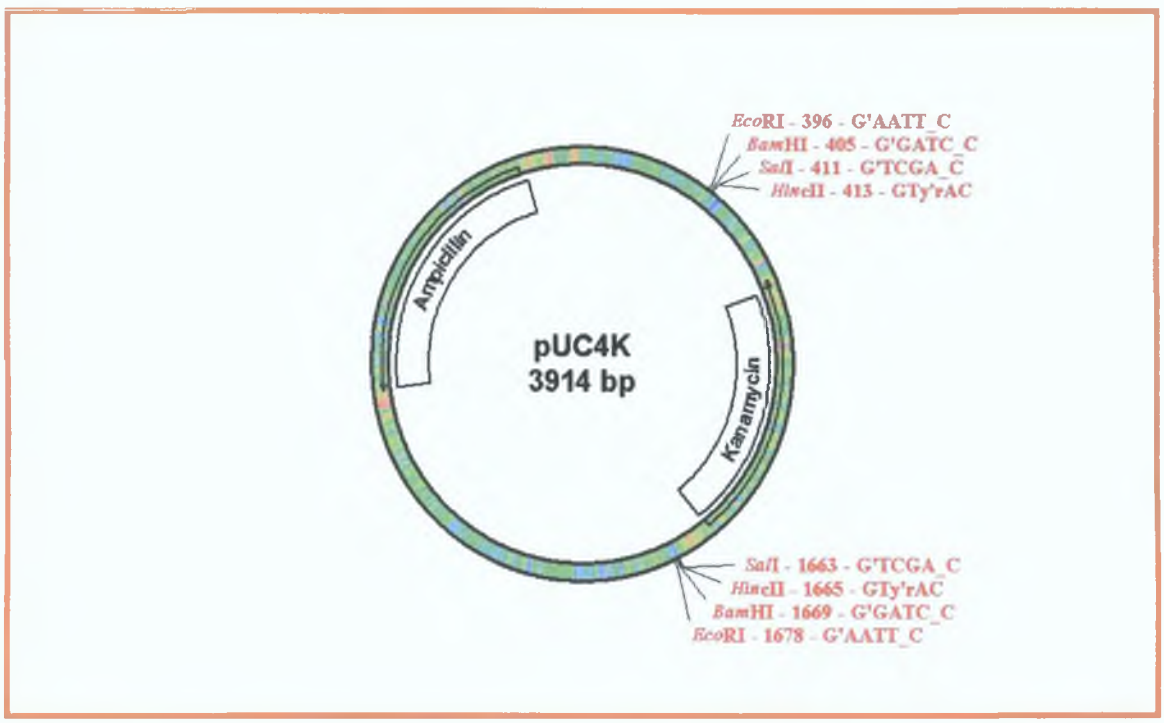


Fig 4.4: Map of pUC4K (Vieira and Messing, 1982) with kanamycin resistance cassette.

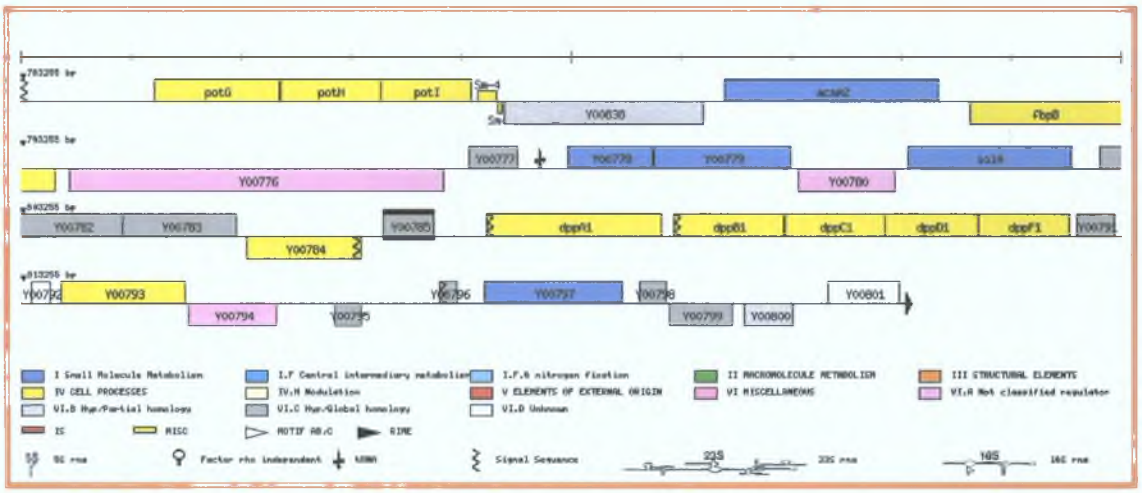


Fig 4.5: *rirA* gene in the *S. meliloti* genome

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 *NcoI* site within the gene into which the kanamycin resistance cassette could be inserted.

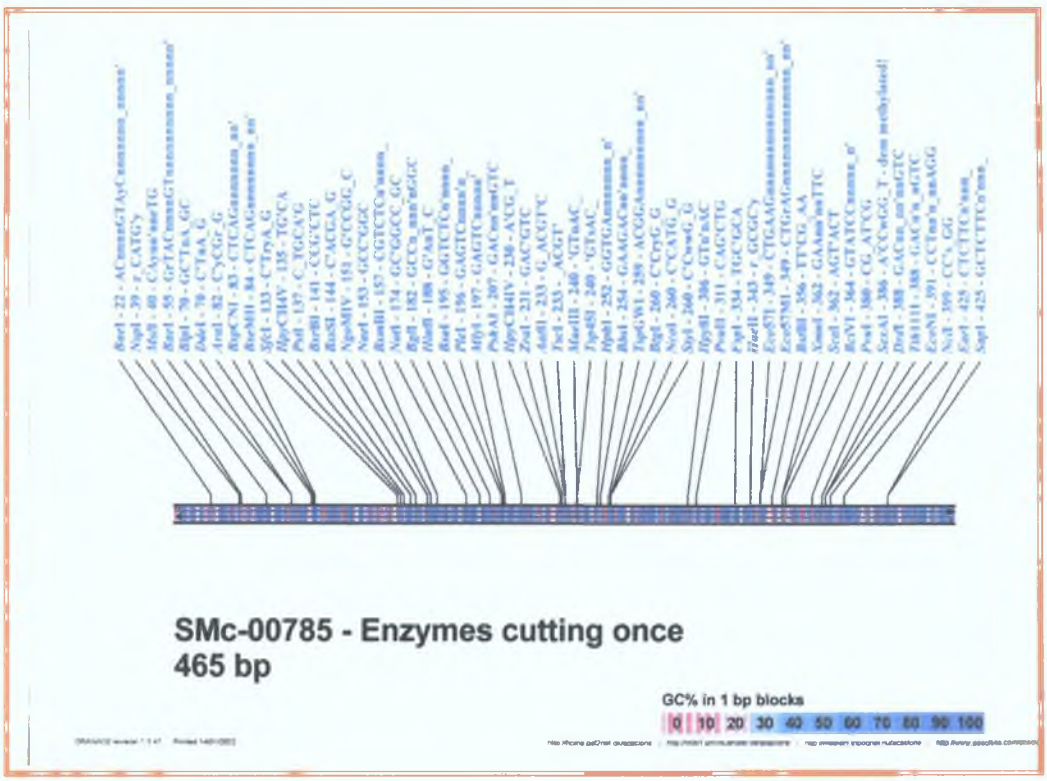


Fig 4 6 Enzymes that only cut the *rirA* gene once

Two primers *rirA*-F and *rirA*-R were designed to amplify an approximatively 2 0 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 *SpeI* site into the PCR product The unique *SpeI* and *XhoI* sites in the PCR product were added to allow for the subsequent directional cloning of the 2 0 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 2 0 Kb PCR product was obtained and cloned into the pCR2 1 vector The 2 0 Kb fragment was restricted from pCR2 1 as an *XhoI/SpeI* 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 summaries the strategy used to construct the final clone, called pRirA200K

PCR Conditions

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

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

***rirA*-F CTCGAG** TCG CCG AGG CCC ATT CCT TCT G

XhoI

***rirA*-R ACTAGT** GAA GTC GGC TGT AAA CGG TAT GCG

SpeI

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.

PCR Conditions
Annealing Temperature 68°C
Annealing Time 1 min
Extension Time 72° C for 3 min

- *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:

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

***KanNcoI*-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

PCR Conditions
Annealing Temperature 64°C
Annealing Time 1 min
Extension Time 72°C for 1.5 min

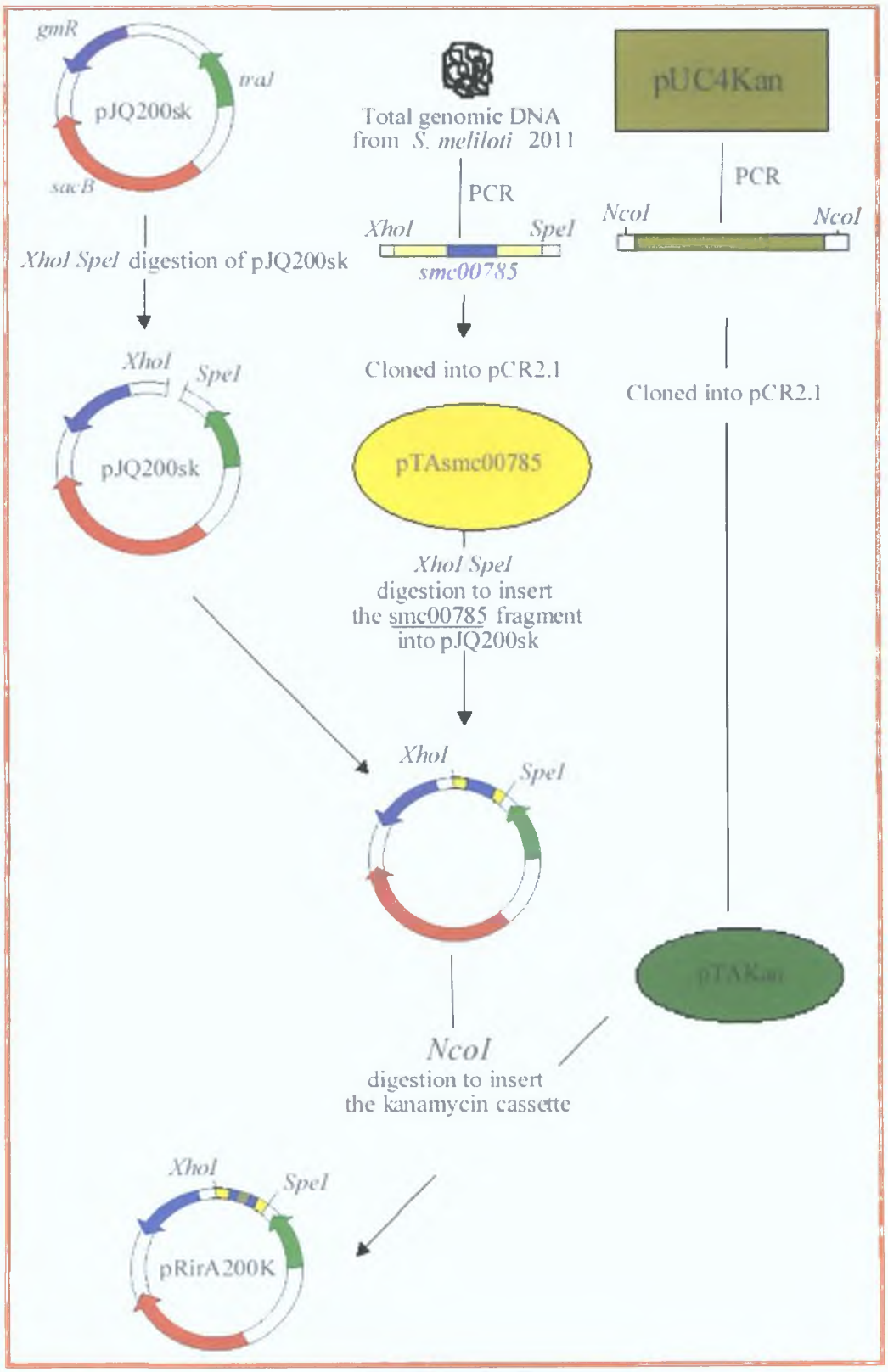


Fig. 4.7: Strategy for the mutation of *S. meliloti rirA* (*smc00785*) gene

The plasmid was introduced into *S 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 *S meliloti* 2011 and from the mutant strain *S 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 *S meliloti* 2011*rirA*2

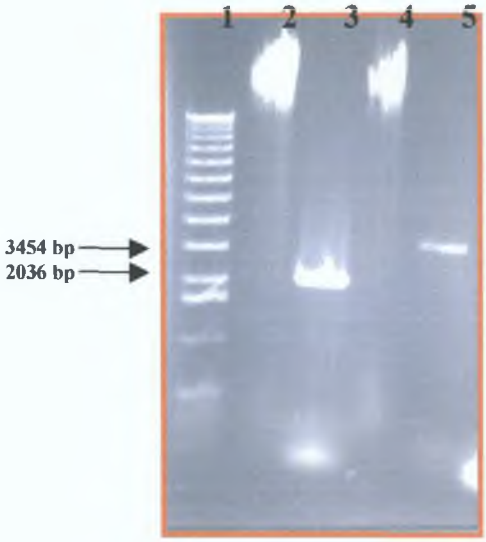


Fig 4.8: PCR to confirm mutation of the chromosomal *rirA* gene

Lane 1: 1 Kb ladder
Lane 2: Chromosomal prep from *S. meliloti* 2011
Lane 3: *rirA* PCR using *rirA*-F and *rirA*-R on *S. meliloti* 2011
Lane 4: Chromosomal prep from *S. meliloti* 2011*rirA*2
Lane 5: *rirA* PCR using *rirA*-F and *rirA*-R on *S. meliloti* 2011*rirA*2

The mutation was also confirmed by Southern hybridisation. The genomic sequence in the region encoding *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 *NcoI* site encoded within a 5.9 Kb *XhoI-XhoI* fragment (Fig 4.9) as an *NcoI* fragment. Digestion of the mutant genomic DNA would generate one fragment with an *XhoI* digestion (Fig 4.10). The plasmid pRirA200K was labeled and used as a probe.

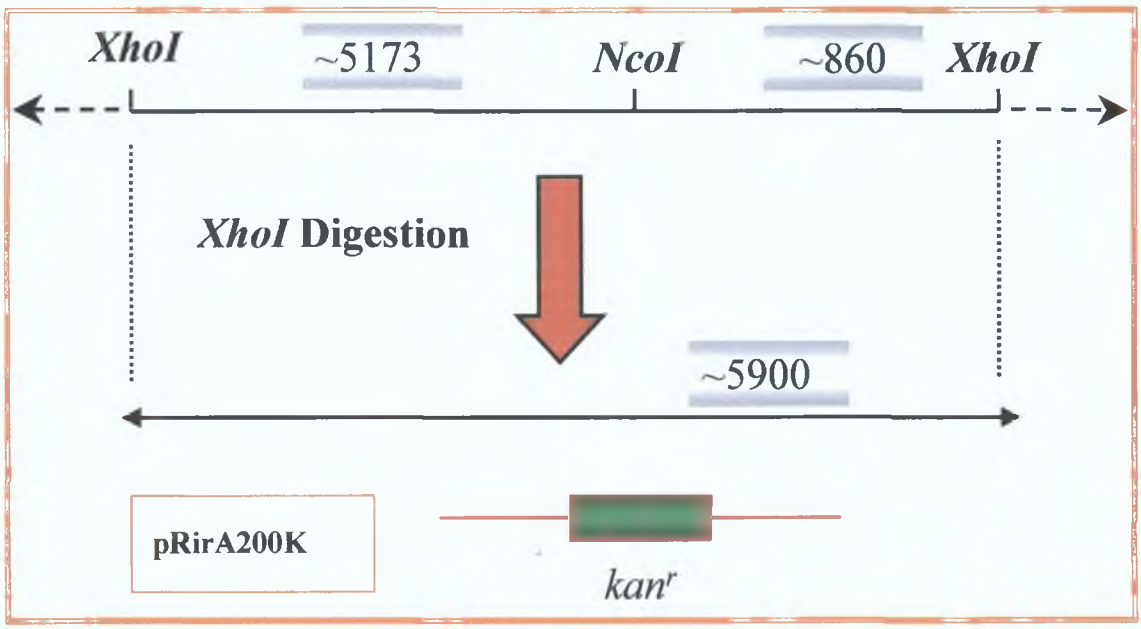


Fig. 4.9: The region encoding *rirA* in *S. meliloti* showing the *XhoI* and *NcoI* restriction sites and the fragment sizes that would hybridise with the pRirA200K probe.

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.

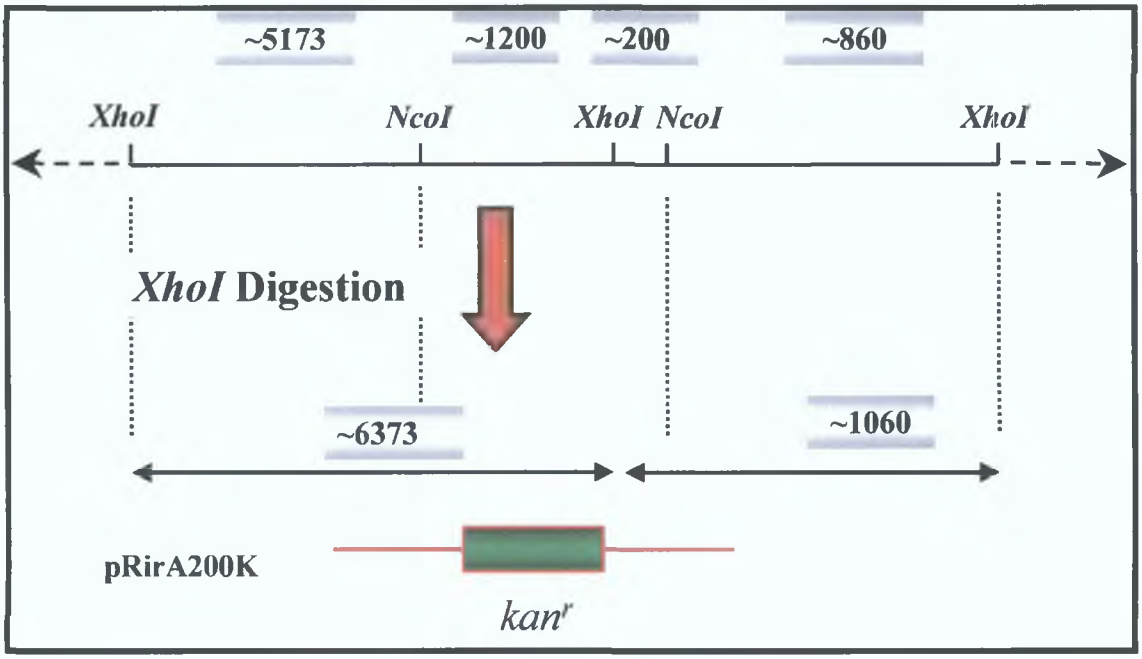


Fig. 4.10: The region encoding *rirA* in a potential mutant showing the *XhoI* and *NcoI* restriction sites and the fragment sizes that would hybridise with the pRirA200K probe.

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 indicated.

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).

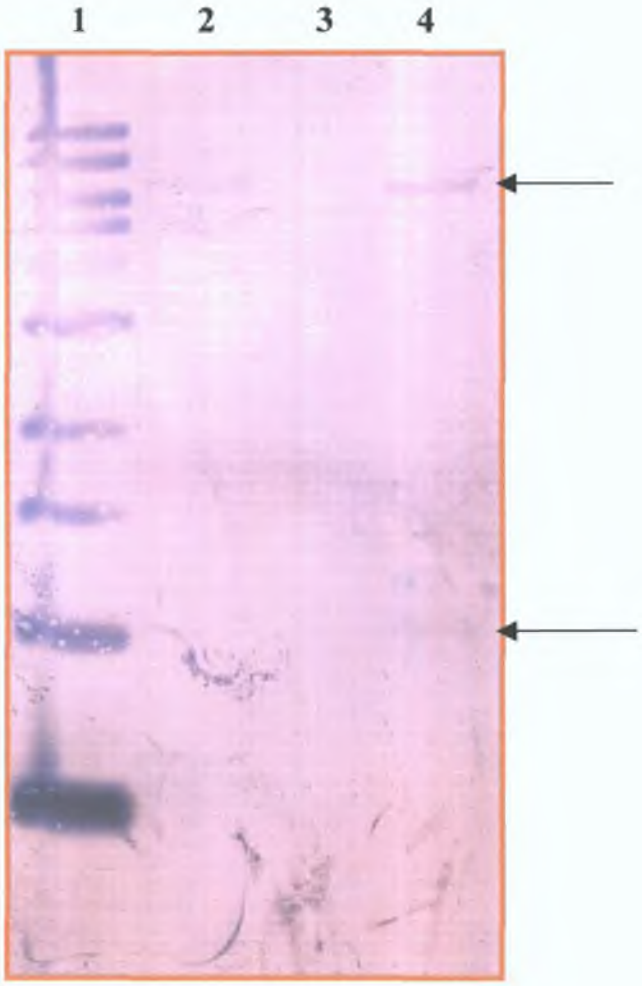


Fig 4. 11: Southern hybridisation analysis of the *S. meliloti* 2011 and *S. meliloti* 2011*rirA*2 confirming the correct insertion of the kanamycin resistance cassette.

Lane 1: 1 Kb Ladder
Lane 2: *S. meliloti* 2011 *XhoI*
Lane 3: none
Lane 4: *S. meliloti* 2011*rirA*2 *XhoI*

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).

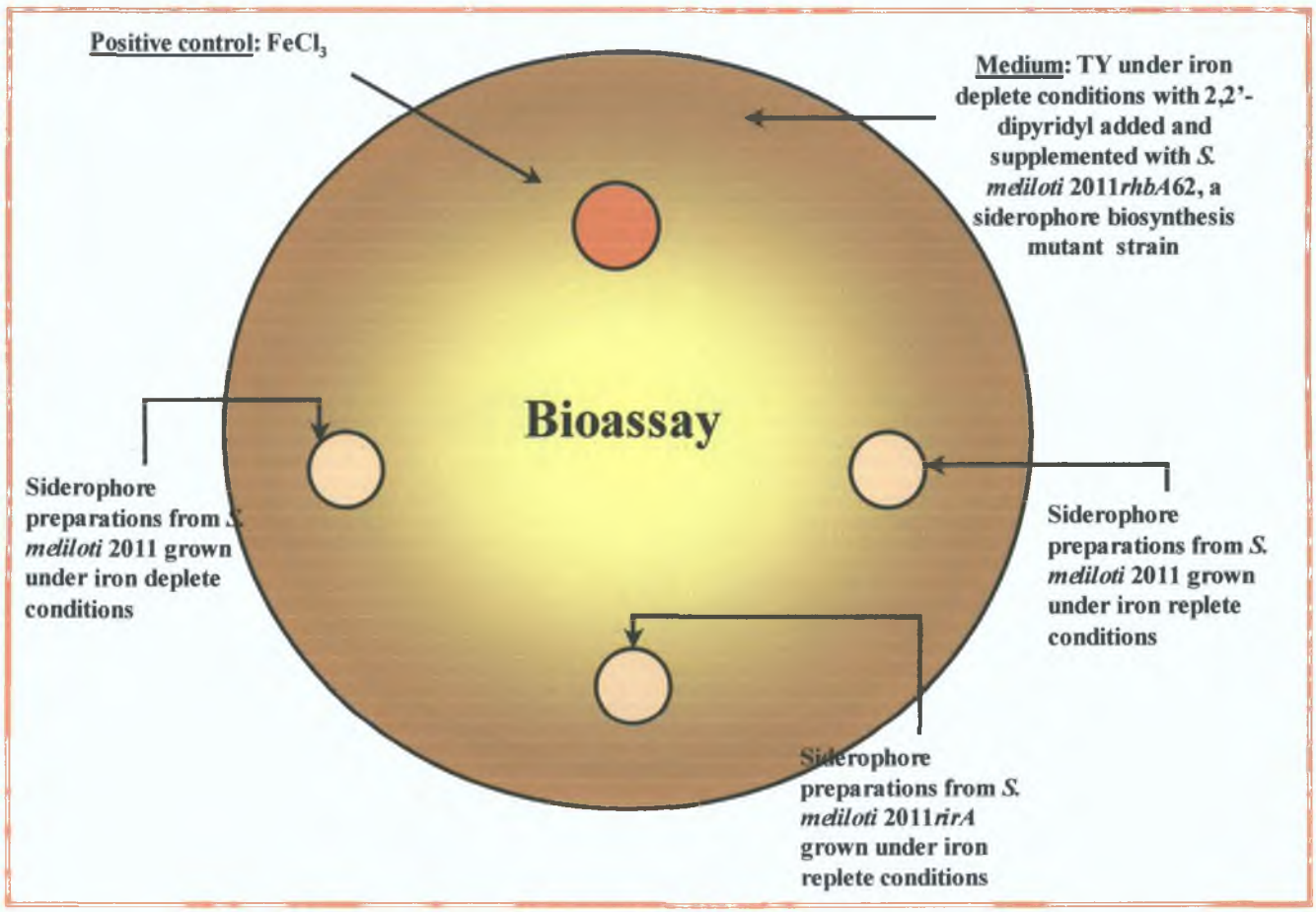


Fig. 4.12: Siderophore Plate Assay

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*rirA*2 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*rhbA*62, a siderophore biosynthesis mutant

4.4.2 Phenotypic analysis of S. meliloti *2011*rirA*2 by the siderophore plate bioassay*

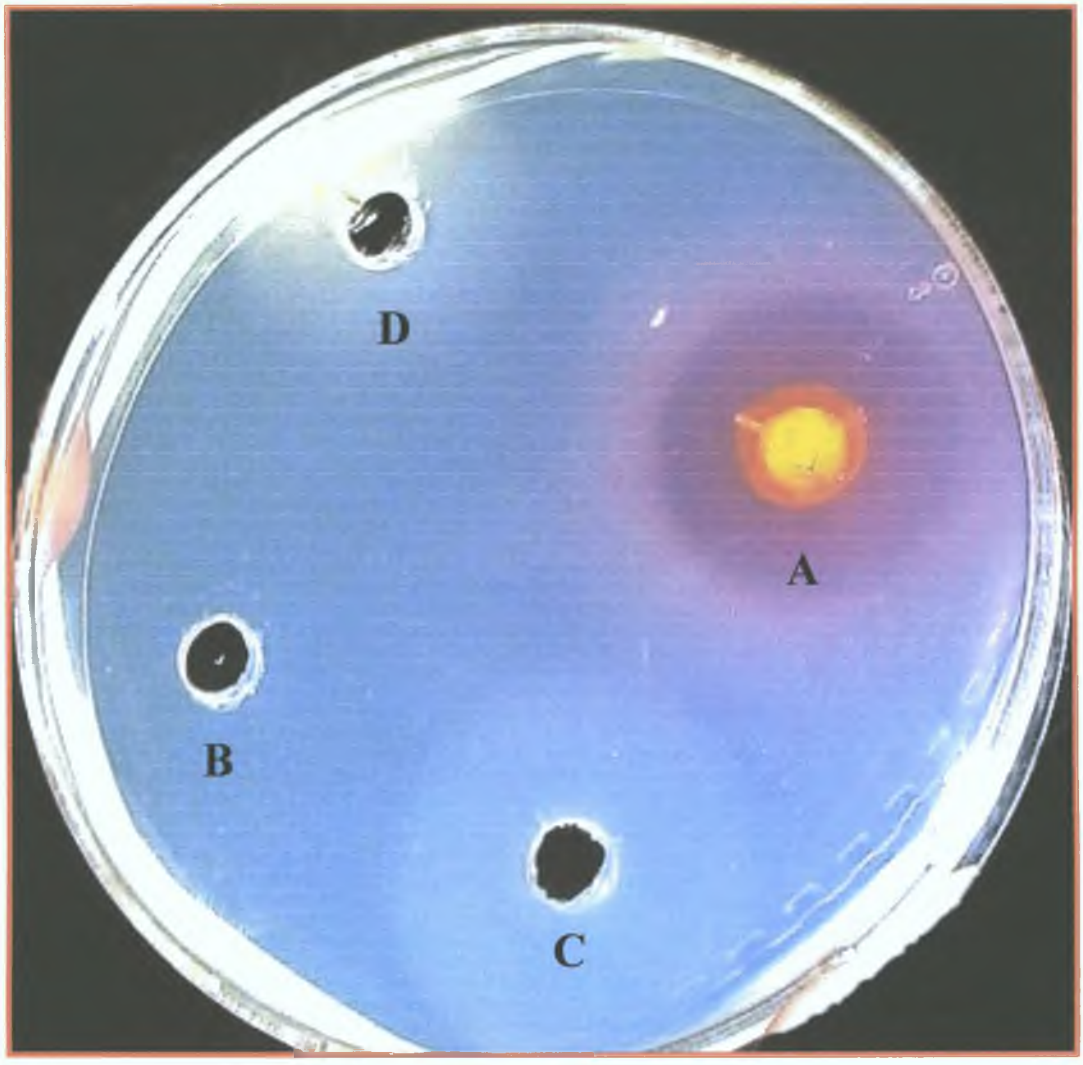


Fig 4.13: Siderophore plate bioassay

A: $FeCl_3$
B: Iron replete conditions, *S. meliloti* 2011 siderophore preparation
C: Iron deplete conditions, *S. meliloti* 2011 siderophore preparation
D: Iron replete conditions, *S. meliloti* 2011*rirA*2 siderophore preparation

As expected in the negative control, the siderophore preparation from iron replete grown *S. meliloti* 2011, no halo of *S. meliloti* 2011*rhbA*62 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*rirA*2 grown under iron replete conditions, a halo of growth surrounded the well implying that *S. meliloti* 2011*rirA*2 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*

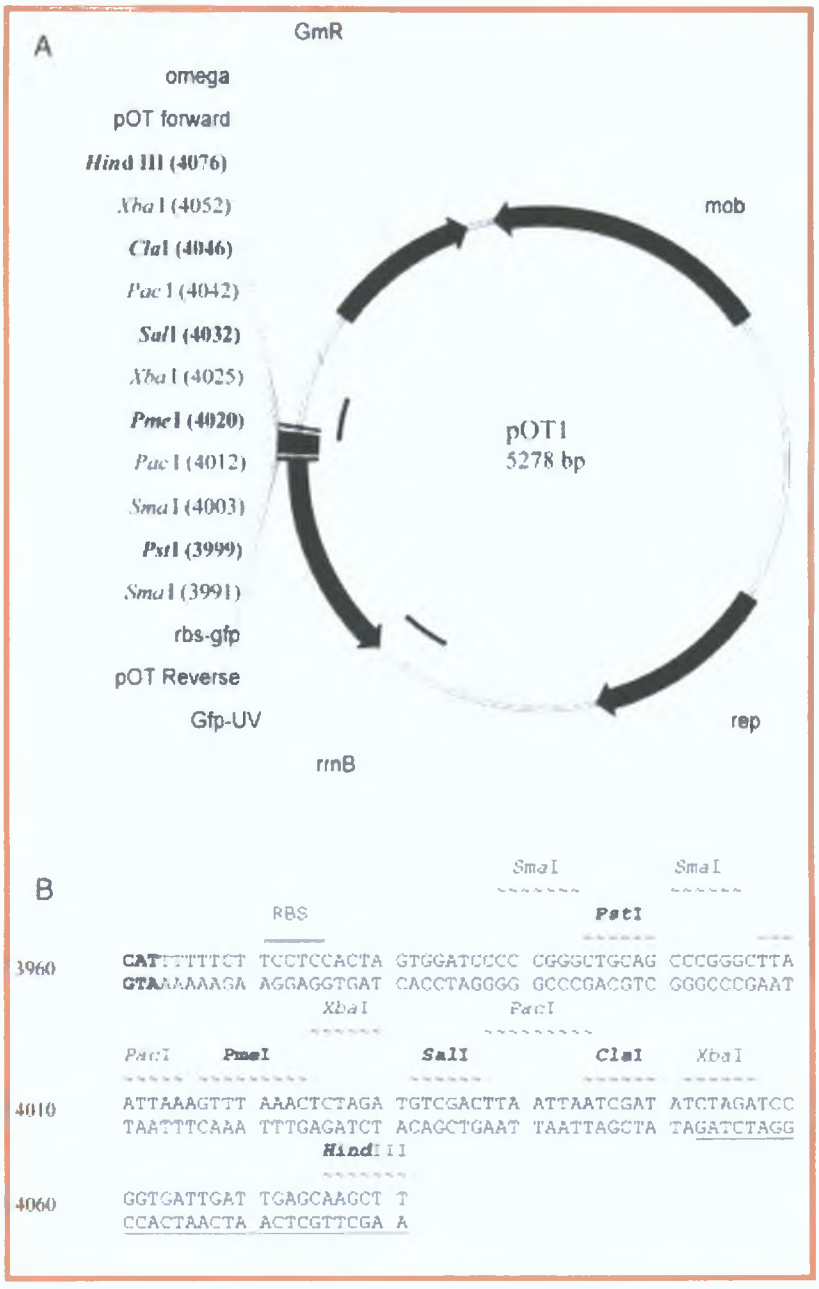


Fig. 4.14: Map of pOT1

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 *rhtXrhbABCDEF* 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).

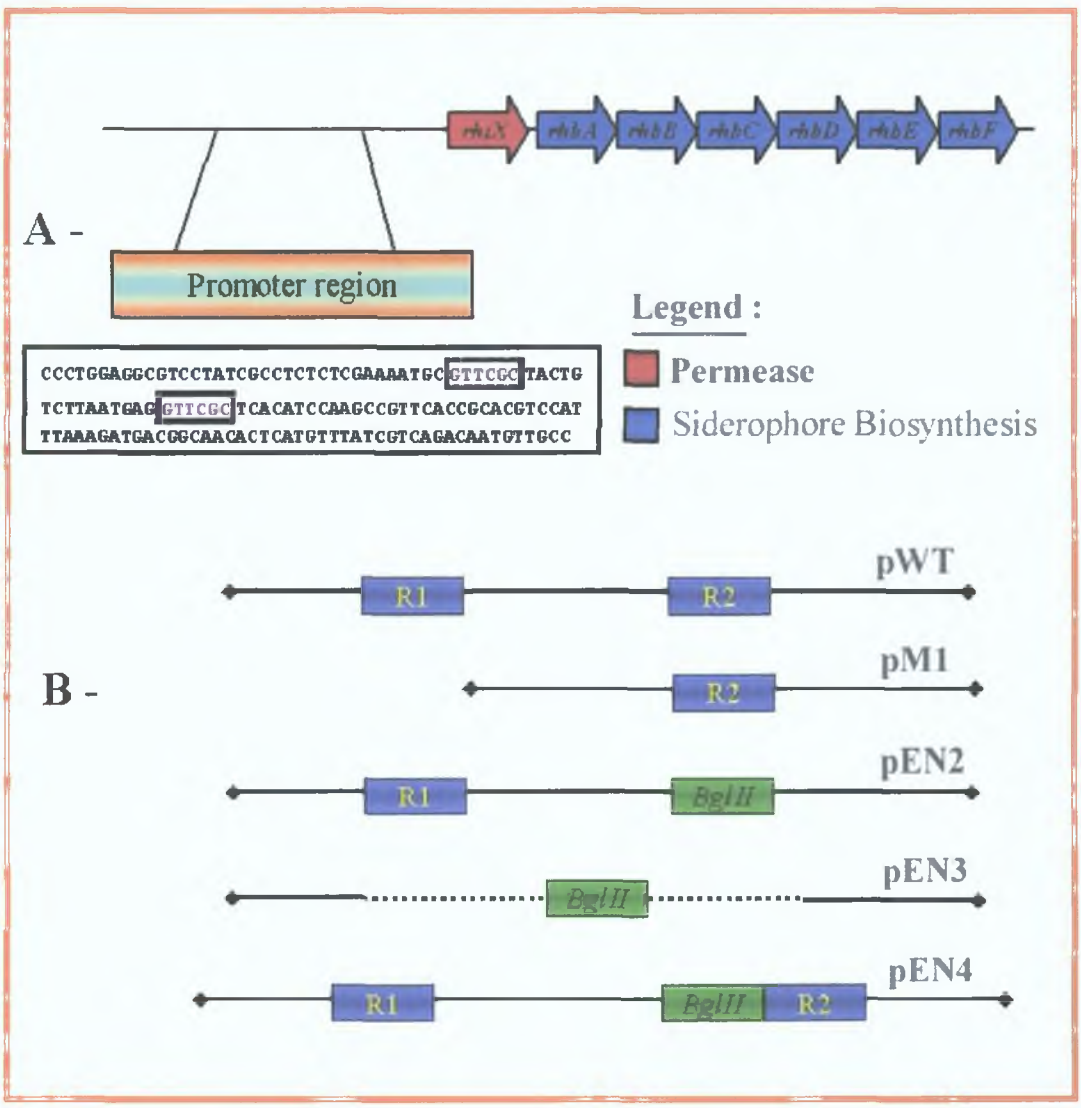


Fig 4.15: Design of the promoter probes
A: The *rhtX* promoter region. The two repeats upstream of *rhtX* are framed. B: Plasmid constructs in which the R1 and R2 repeats have been altered.

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 16 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 *NcoI – 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 *NcoI-EcoRI* restricted pOT1 vector and the transformants were then selected on medium containing gentamicin

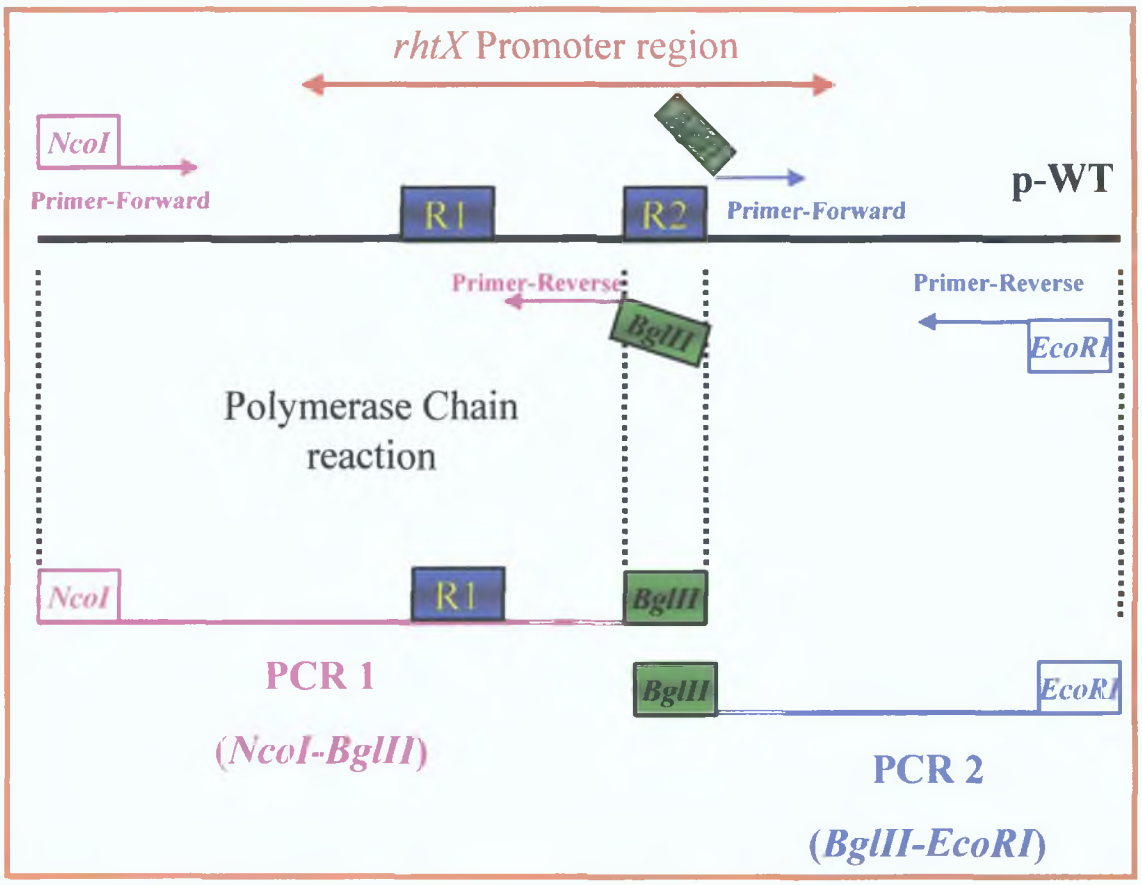


Fig 4.16: The PCRs performed on pWT for the cloning of pEN2

The following primers were designed for the amplification of the *rhtX* promoter region and for the mutagenesis of that region:

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

HindIII

AAGCTTCCCT GGAGGCGTCC TATCGCCTCT CTCGAAAATG CGTTCGCTAC

TGTCTTAATG AGGTTCGCTC ACATCCAAGC CGTTCACCGC ACGTCCATTT

AAAGATGACG GCAACACTCA TGTTTATCGT CAGACAATGT TGCCTGCAG

PstI

F-rhrAWT:

CCC AAGCTT CCC TGG AGG CGT CCT ATC GCC

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*.

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

F-rhrAM1:

TTT AAGCTT TAC TGT CTT AAT GAG GTT CGC TCA C

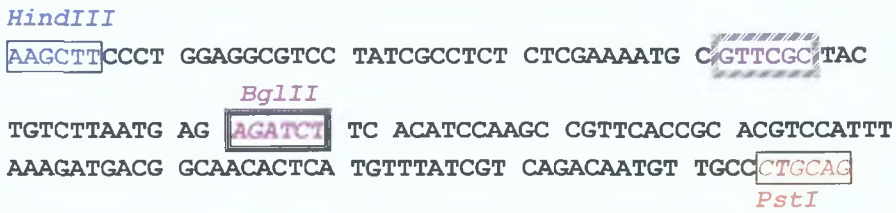
R-rhrAWT

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*.

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

Primers for the construction of p-EN2: Promoter probe where the proximal repeat of the *rhtX promoter* region of p-WT is removed:



♦ PCR 1:

F-NcoIpOT1:

CA**GT** CCATGG GCA AAT GGG ATT GGC

R-BglIIR2 :

GA AGATCT CTC ATT AAG ACA GTA GCG AAC GC

♦ PCR 2:

F-BglIIR2:

GA AGATCT TCA CAT CCA AGC CGT TCA CCG C

R-EcoRIpOT-1:

CG GAATTC ATT ATT TGT AGA GCT CAT CC

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

AAGCTTCCCT GGAGGCGTCC TATCGCCTCT CTCGAAAATG C AGATCT TC
ACATCCAAGC CGTTCACCGC ACGTCCATTT AAAGATGACG GCAACACTCA
TGTTTATCGT CAGACAATGT TGCCCTGCAG
PstI

- PCR 1:

F-NcoIpOT-1:

CAGT CCATGG GCA AAT GGG ATT GGC

R-BglIIR1R2:

GA AGATCT GCA TTT TCG AGA GAG GCG ATA GG

- PCR 2:

F-BglIIR2:

GA AGATCT TCA CAT CCA AGC CGT TCA CCG C

R-EcoRIpOT-1:

CG GAATTC ATT ATT TGT AGA GCT CAT CC

Primers for the construction of p-EN4: Promoter probe where an additional six bases where added upstream of the proximal repeat of the *rhtX* promoter region of p-WT:

♦ PCR 1:

F-NcoIpOT-1:

CA**GT** CCATGG GCA AAT GGG ATT GGC

R-BglIIR2:

GA AGATCT TCA CAT CCA AGC CGT TCA CCG C

♦ PCR 2:

F-BglII+6:

GA AGATCT GTT CGC TCA CAT CCA AGC CGT TC

R-EcoRIpOT-1:

CG GAATTC 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 *rhtXrhbABCDEF*.

PCR Conditions
Annealing Temperature 68°C
Annealing Time 1 min
Extension Time 72°C for 1 min

The constructed plasmids were then mobilised into three different strains:

- *S. meliloti* 2011
- *S. meliloti* 2011*rirA*2, a *rirA* mutant
- *S. meliloti* 2011*rhrA*26, 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:

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

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 rhtXrhbABCDEF *promoter sequences and measured in the wild type,* rirA *and* rhrA*26 backgrounds*

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

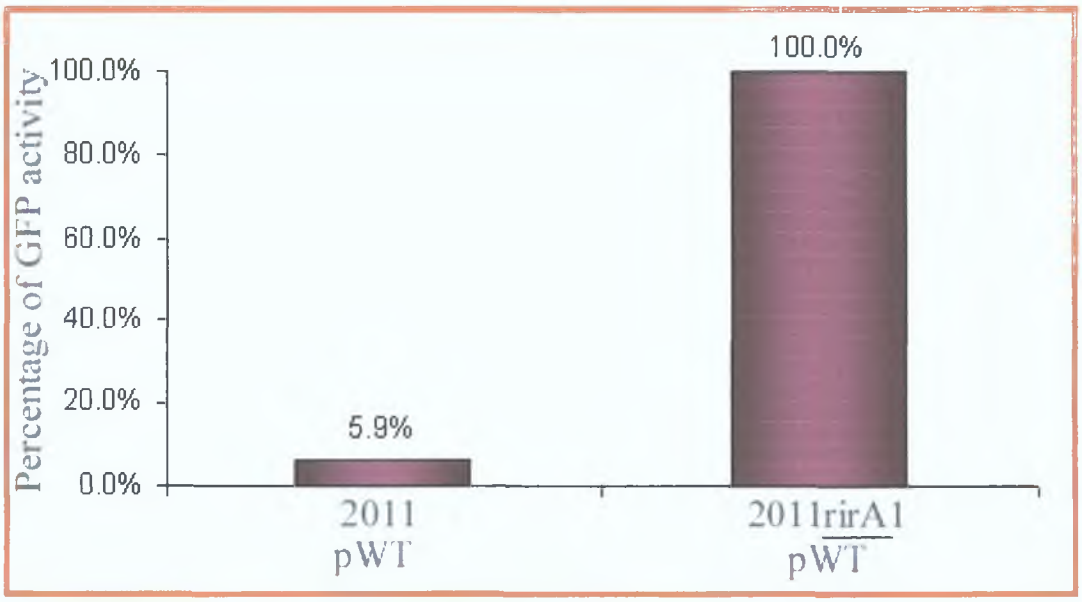


Fig 4.17: GFP activity of *S. meliloti* 2011[pWT] vs. 2011*rirA*2 [pWT] under iron replete conditions.

The results indicate an increased level of the reporter gene expression in the *rirA*2 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 *rirA*2 [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).

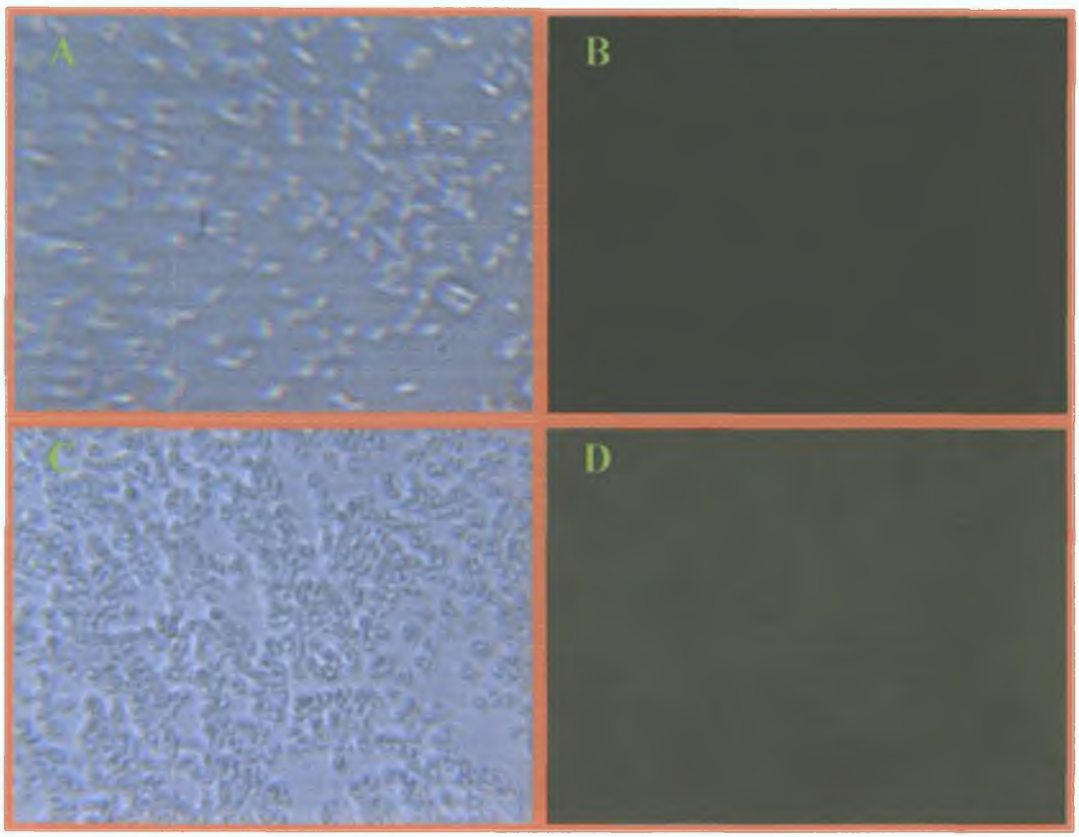


Fig 4.18: Culture of *S. meliloti* 2011 [pOT1] (A and B) and *S. meliloti* 2011 [pWT] (C and D) under bright light to confirm the presence of the bacteria (A and C) and UV light for green fluorescent protein (B and D). Magnification 1000 X.

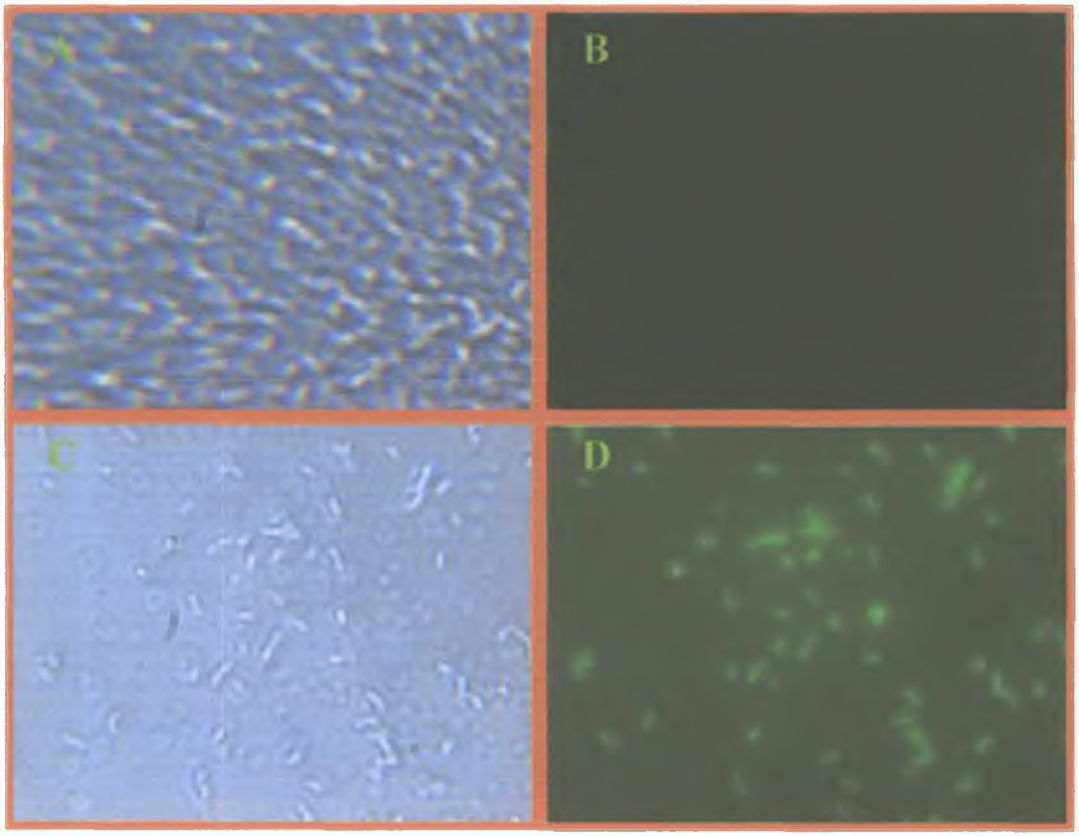


Fig 4.19: Culture of *S. meliloti* 2011*rirA*2 [pOT1] (A and B) and *S. meliloti* 2011*rirA*2 [pWT] (C and D) under bright light to confirm the presence of the bacteria (A and C) and UV light for green fluorescent protein (B and D). Magnification 1000 X.

These findings demonstrate that under iron replete conditions, the *gfp* gene is only expressed in the *rirA2* mutant *S. meliloti* 2011*rirA*2 [pWT]. The plasmid pWT contains the iron-responsive *rhtX* promoter region, fused to a *gfp* reporter gene in the wide-host-range promoter-probe plasmid pOT1. This suggests that the *S. meliloti rirA* mutant is defective in the iron dependent repression of the expression of the operon *rhtXrhbABCDEF* 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 *S. meliloti* 2011 were grown under iron replete conditions until late exponential phase and then examined for GFP activity.

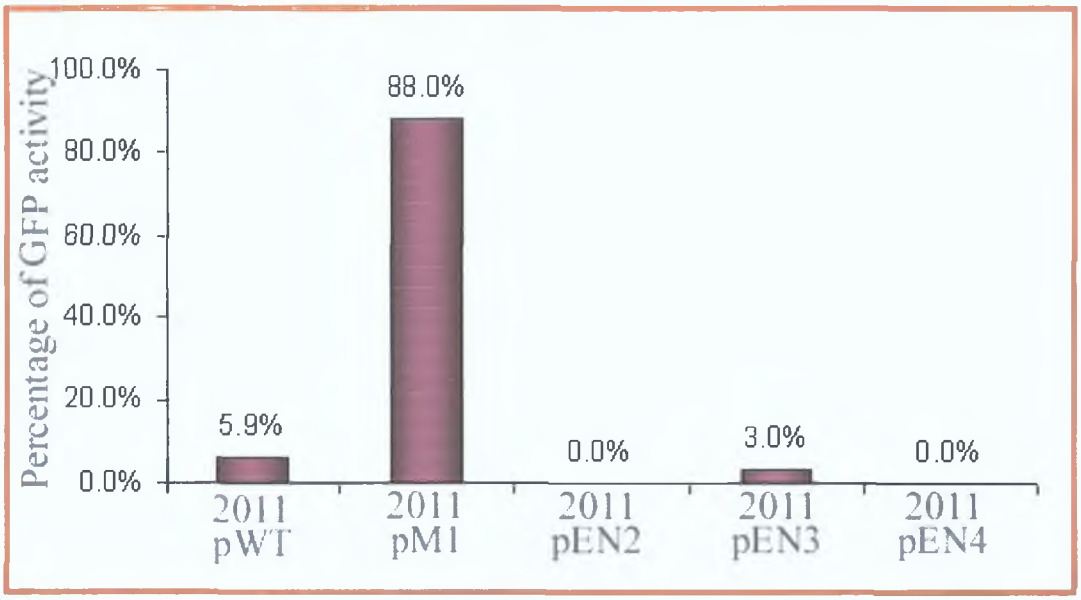


Fig 4.20: GFP activity of the different promoter probes under iron replete conditions in *S. meliloti* 2011.
(because it has the higher level relative level of fluorescence under iron replete conditions: 100% was given to 2011*rirA*2 pWT)

GFP activity was only detected in *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 *S. meliloti* 2011 [pM1] compared to *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 *rhtXrhbABCDEF* 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 *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.

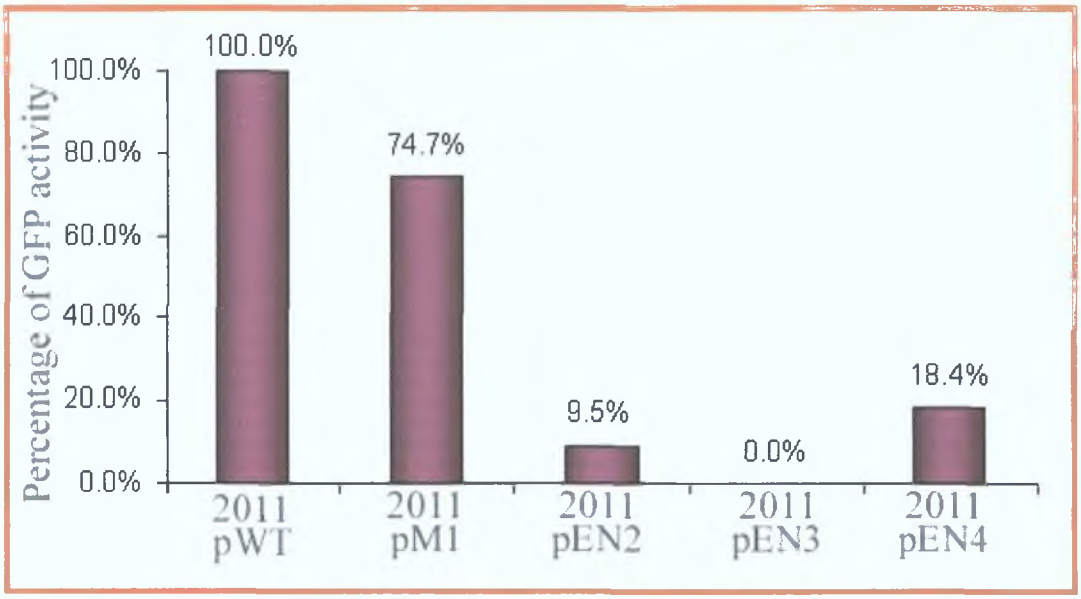


Fig 4.21: GFP activity of the mutated promoter fusions under iron deplete conditions in *S. meliloti* 2011. The negative control was *S. meliloti* 2011 2011*rhrA*26 for which none of the promoter probes gave any GFP activity.

These findings demonstrate that under iron deplete conditions, the *gfp* gene is only highly expressed in *S. meliloti* 2011 [pWT] and *S. meliloti* 2011 [pM1]. The plasmid pWT contains the iron responsive *rhtXrhbABCDEF* promoter region, fused to a *gfp* reporter gene in the wide-host-range promoter-probe plasmid pOT1 and the plasmid pM1 contains the iron responsive *rhtX* promoter region, fused to a *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 house keeping 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

Gene	Primers
rhbA	RhbA-F: ATG CCG GCC GAT TTA GCC RhbA-R: TCG CGT CTT TCC TGT CGG
rhtA	RhtA-F: CTATGGAATTGGCAACTACTC RhtA-R: CGATGATCTCAACGGCAAGC
rhrA	RhrA-F: TGC CAG CGA CAG GGA AAC G RhrA-R: ATG GAG ACA ATC CGA CCG
dppA1	dppA1-F: CAC TAC TCT CTT GGC 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: TGCTCAACCGGCATCATCGCCTGGC Smc02726-R: CGCGACGATCTTCTTCAGCACGGTC
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 CCG

The primers' specifities 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 Tn*5* 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.

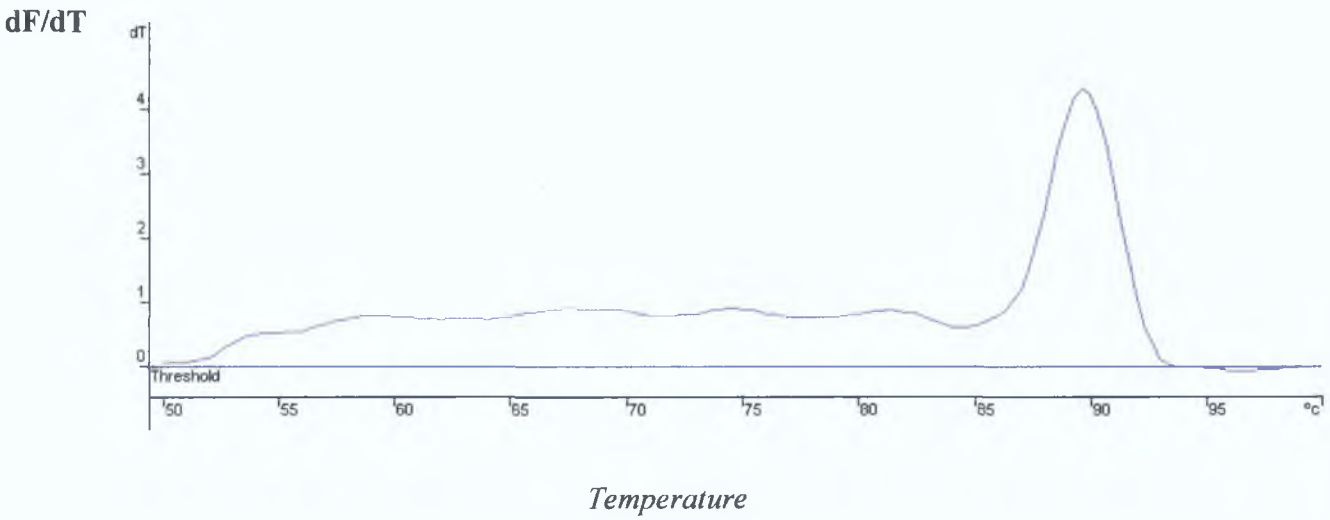


Fig 4.22: Melting temperature curve of the *rirA* gene of *S. meliloti* grown under iron replete conditions

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

Δ Ct= endogenous Ct – Gene of interest Ct

ΔΔCt = ΔCt of sample - ΔCt of calibrator

Amount of target normalised to a control and relative to a calibrator = 2(ΔΔCt)

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 *rirA* itself were determined. To do this, a real-time RT-PCR was performed comparing the level of mRNA of *rirA* in *S. meliloti* under iron deplete and replete conditions. In this experiment, the housekeeping gene chosen was *npt*.

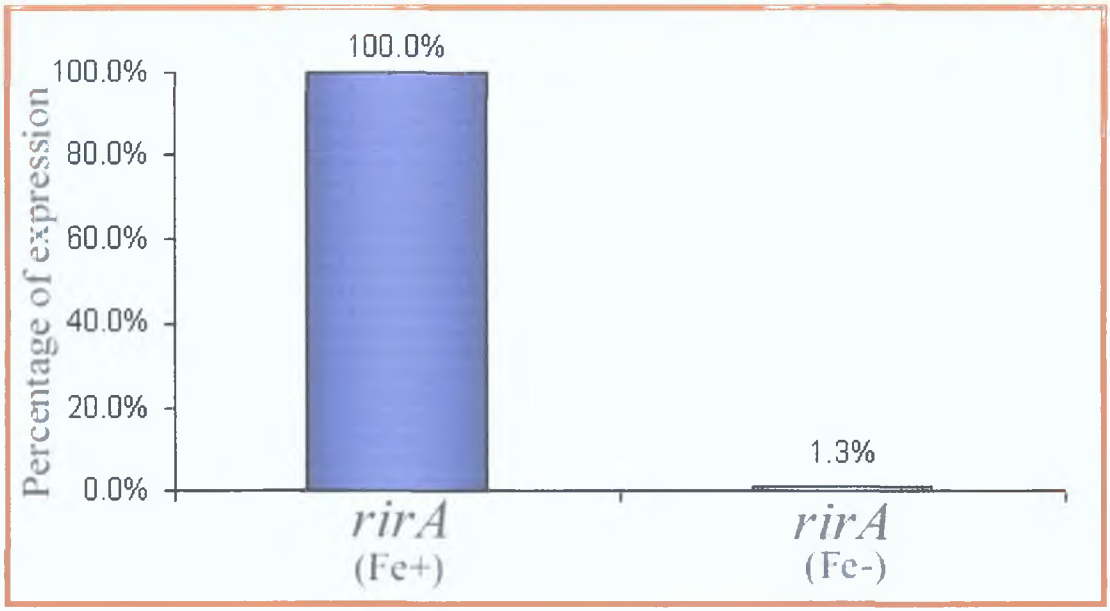


Fig 4.24: *In vivo* analysis of the iron regulation of *rirA* by Real-Time PCR
(Fe+): Iron replete condition (Fe-): Iron deplete condition

The real-time RT-PCR (Fig 4.24) clearly showed that the expression of *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 rhbA *and* rhtA *as detected by real time RT-PCR.*

The effect of iron was also determined on the expression of *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 *rhbA* and *rhtA,* which are genes involved in the siderophore mediated iron uptake system under iron deplete and replete conditions in *S. meliloti* 2011. In this experiment, the housekeeping gene chosen was *npt*.

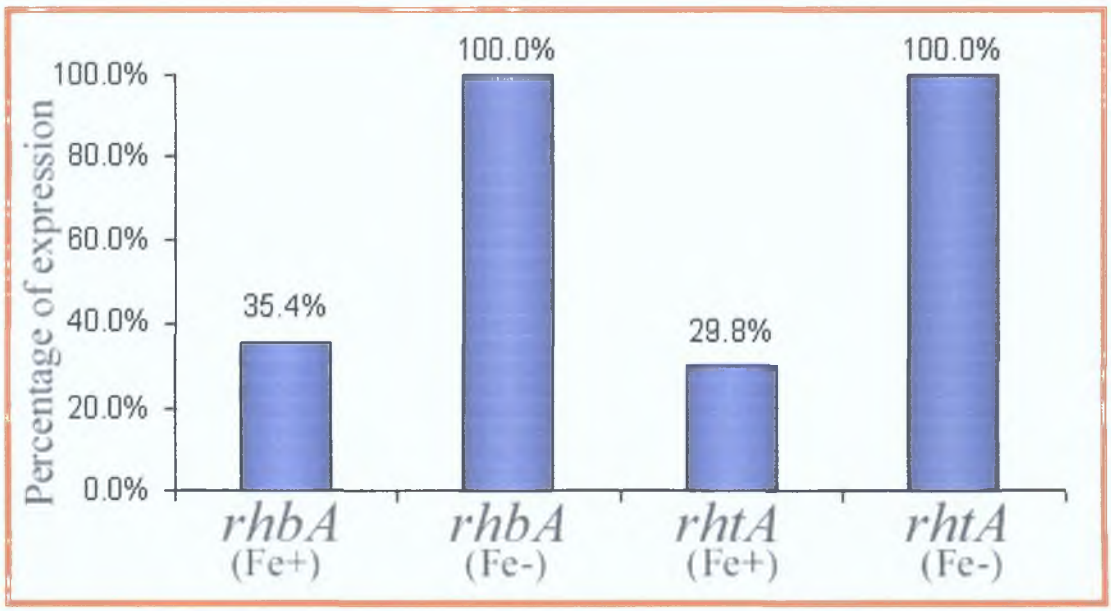


Fig 4.25: *In vivo* analysis of the iron regulation of *rhbA* and *rhtA* by Real-Time PCR
(Fe+): Iron replete condition (Fe-): Iron deplete condition

The real-time RT-PCR (Fig 4.25) confirmed that the expression of the biosynthesis gene *rhbA* and the gene encoding the rhizobactin 1021 outer membrane receptor, *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*rirA*2 using *16S rRNA* as the house keeping gene.

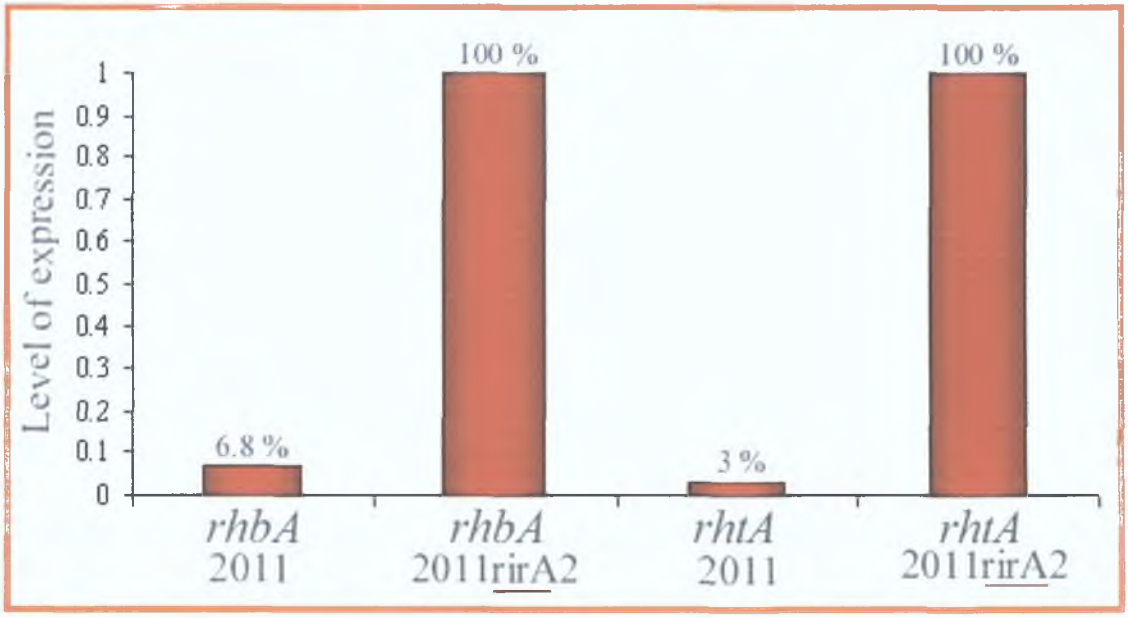


Fig 4.26: *In vivo* analysis of RirA regulation of *rhbA* and *rhtA* in *S. meliloti* 2011 by Real-Time PCR

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 *rirA*2 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 2011*rirA*2. 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 *rirA*2 mutant.

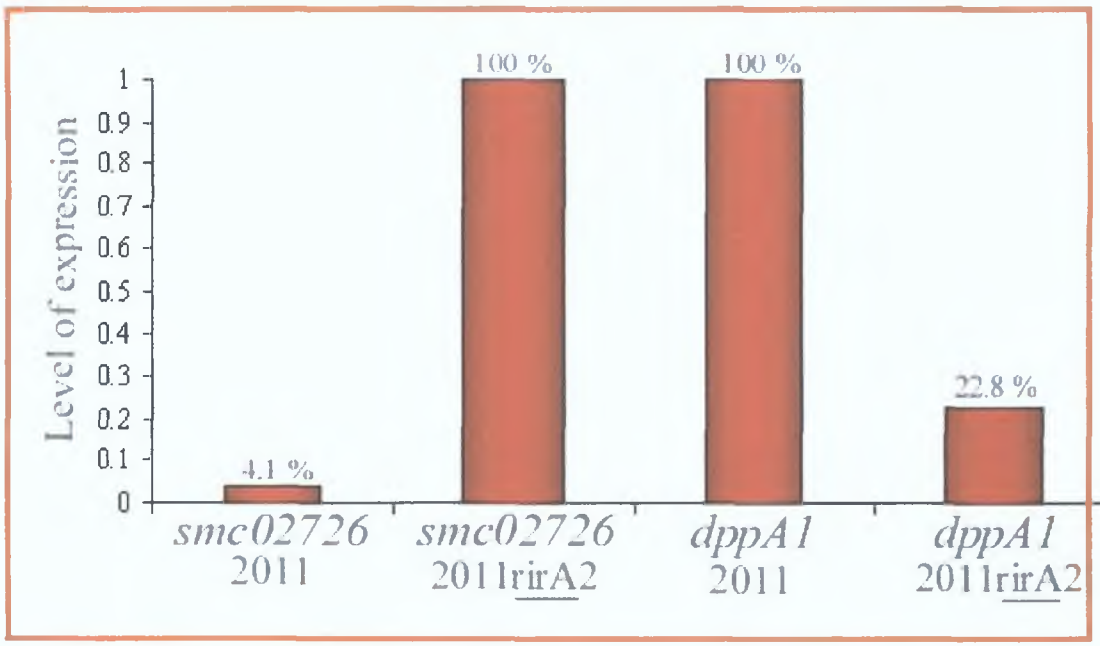


Fig 4.27: *In vivo* analysis of RirA regulation of *dppA1* and *smc02726* in *S. meliloti* 2011 by Real-Time PCR

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 *rirA*2 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 *rhrA* itself were determined. To do this, a real-time RT-PCR was performed comparing the level of mRNA of *rhrA* in *S. meliloti* under iron deplete and replete conditions. In this experiment, the housekeeping gene chosen was *npt*.

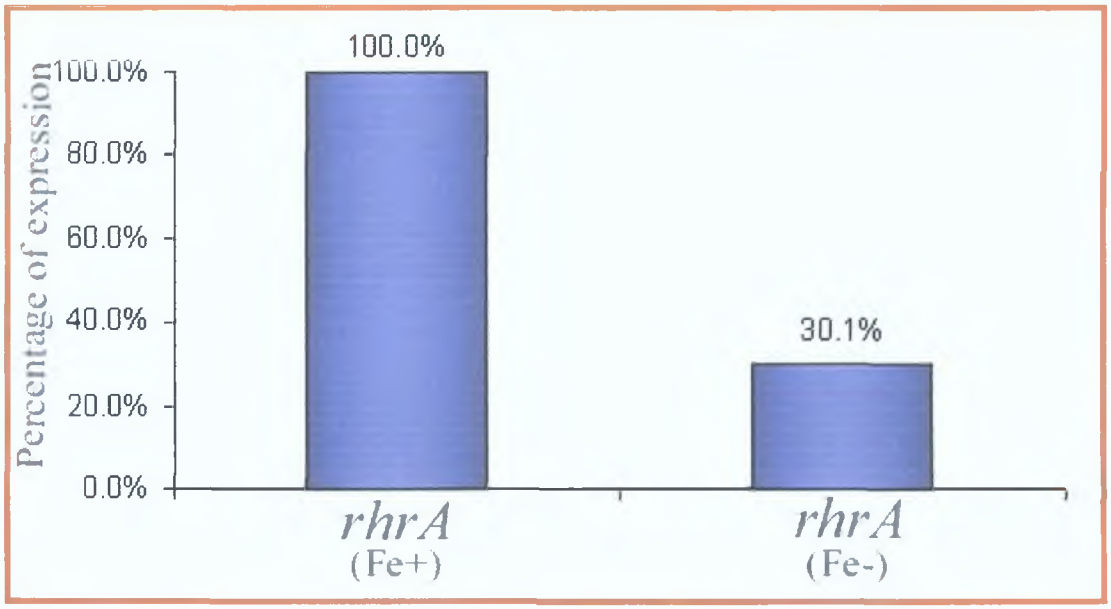


Fig 4.28: Analysis of the iron regulation of *rhrA* by Real-Time PCR
(Fe+): Iron replete condition (Fe-): Iron deplete condition

The real-time RT-PCR (Fig 4.28) showed that the *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.

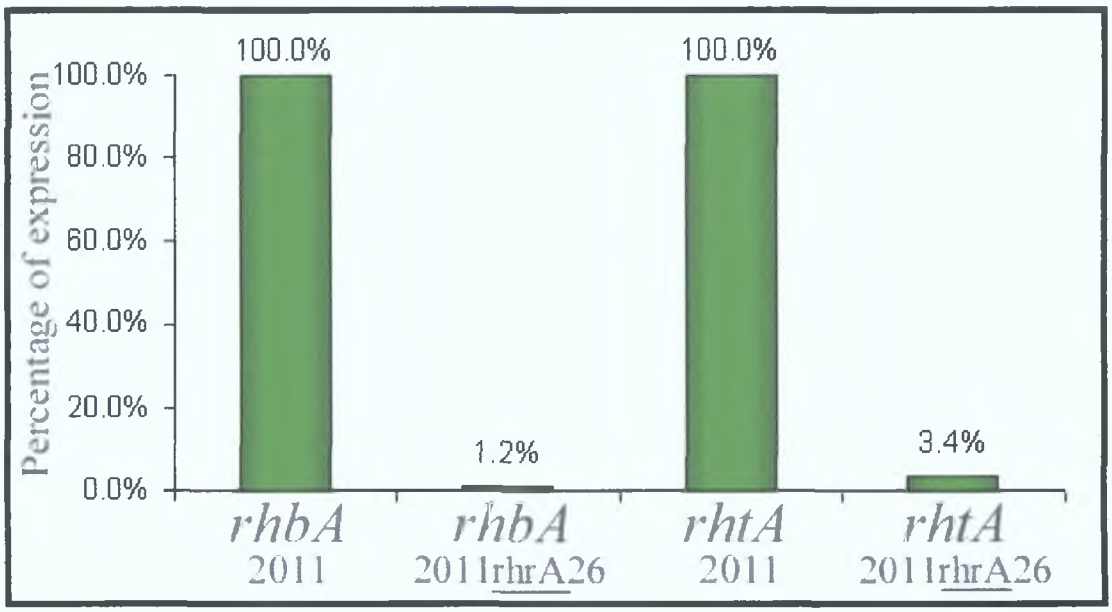


Fig 4.29: *In vivo* analysis of RhrA regulation of *rhbA* and *rhtA* in *S. meliloti* 2011 by Real-Time PCR

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 *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 solubity 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 *S meliloti rhrA* gene, which encodes a 35 KDa protein, was cloned using the same approach as for the *S meliloti fur* homologue (Chapter 3) The gene was amplified by PCR from genomic DNA from *S meliloti* 2011 *NcoI* and *BamHI* sites were incorporated into the forward and reverse primers respectively The amplified fragment extends from the start codon of *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 *Nco*I-*BamH*I fragment carrying the entire PCR-generated fragment was subsequently sub cloned into the expression vector pQE60 This recombinant plasmid, designated pRhrA60, was used to transform *E coli* XL10 gold and Rosetta Blue

The strategy of the *rhrA* cloning into pQE60 is summarised in Fig 4 30

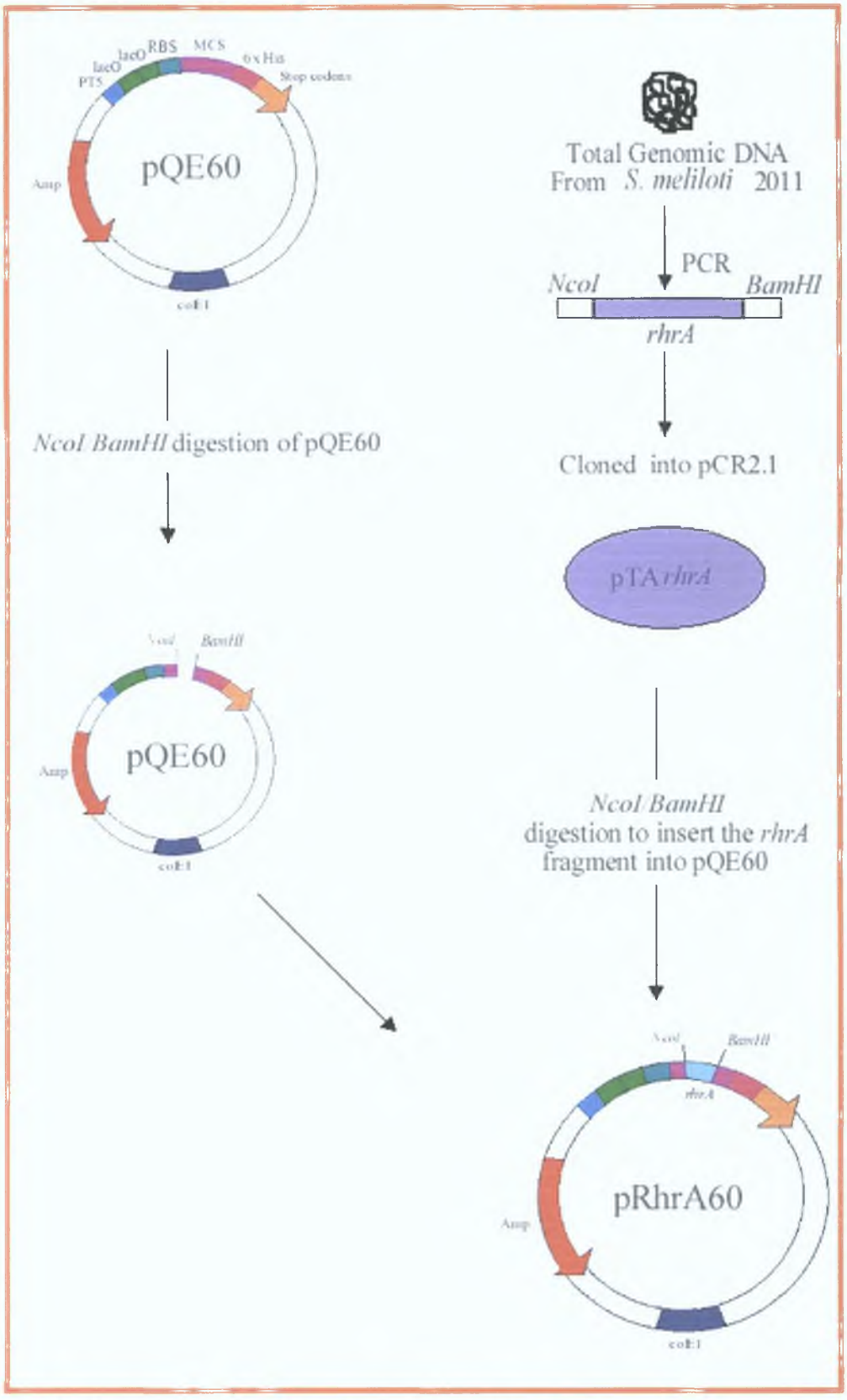


Fig 4.30: Strategy of the *rhrA* cloning into pQE60

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.

PCR Conditions
Annealing Temperature 55°C
Annealing Time 1 min
Extension Time 72°C for 1 min

Problems were encountered with the over expression of *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 *E coli* Rosetta Blue was used as it is designed to enhance the expression of proteins that contain codons rarely used in *E coli* It supplies tRNAs for 6 rare codons, AUA, AGG, AGA, CUA, CCC, GGA, on a compatible chloramphenicol-resistant plasmid called pRARE An analysis of *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 determinate the optimal conditions for high yields of protein

It was determined that the optimised protocol for the overexpression of RhrA was as follows *E coli* Rosetta Blue carrying pRhrA60 was inoculated into LB containing 100 µg/ml of ampicilhn (to maintain the pRhrA60 plasmid) and 30 µg/ml of chloramphenicol (to maintain the pRARE plasmid) and were grown at 30°C until the 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.

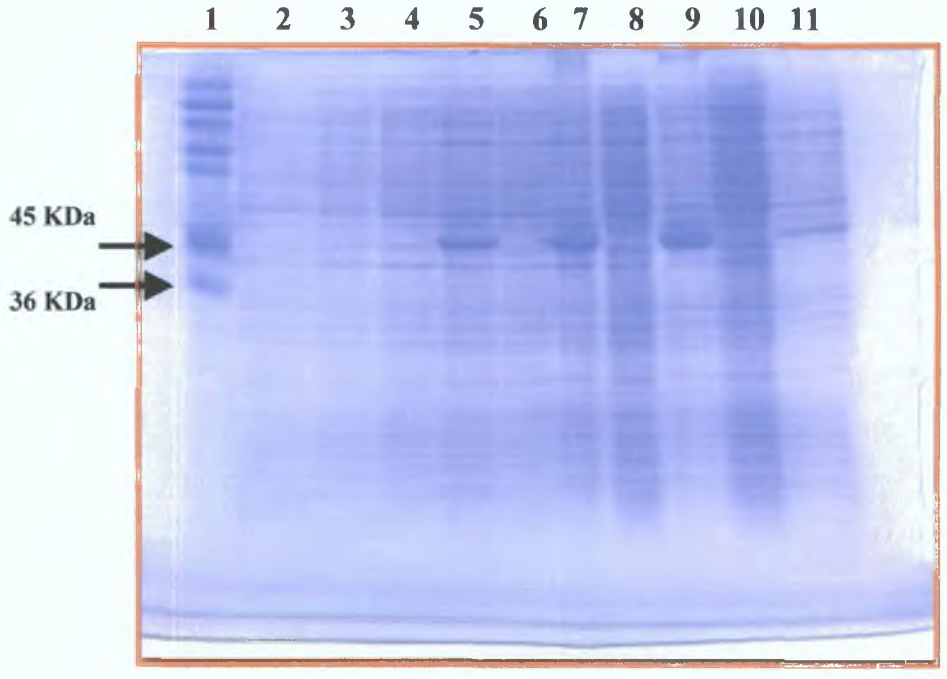


Fig 4.31: 15% SDS polyacrylamide gel electrophoresis of extract of cells overexpressing RhrA prepared under denaturing conditions

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.

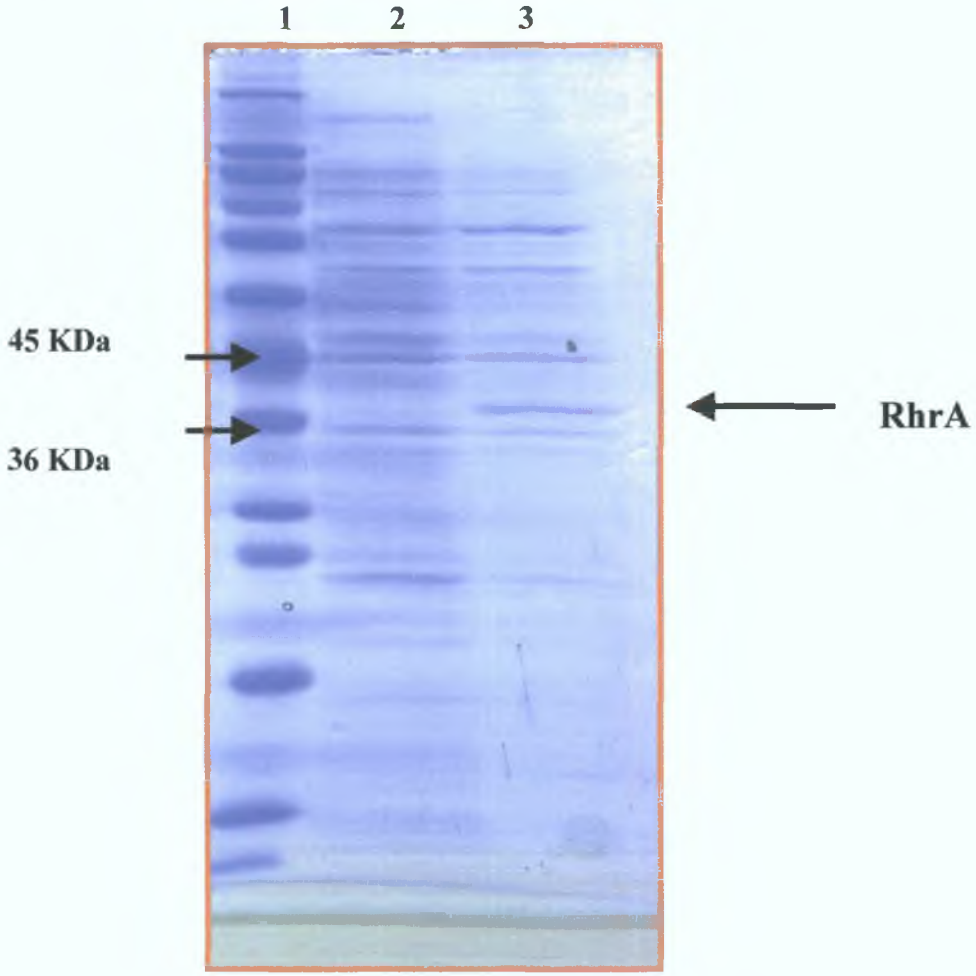


Fig 4.32: 15% SDS polyacrylamide gel electrophoresis of extracts containing RhrA protein that had been prepared under native conditions

Lane 1: ladder
Lane2: 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*

CGGGATCCCCTATCGCCTCTCTCGAAAATGCGTTCGCTACTGTCT TAATGAGGTTCGCTCACATCCAAGCCGTTCACCGCACGTCCATTT AAAGATGACGGCAACACTCATGTTTATCGTCAGACAATGTTGCCG GGCAGTGGCAGTTTTCGGGATCCCG

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*

CGGGATCCGTCGTGCGCCAGCCTTTCCTGTTGACGTTCGCATGCG TCCAAATGAGGTTCGCCATTATCCAAGCGGCGAACACCCTTAGCC CATAAAACATGACTTAAATAGTCTTGTATTGGCAATTTGCCCGCC CACCGGCAGCGGCAATTGTTTTCTGGTGCGCAGGGGGCGTTATG 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).

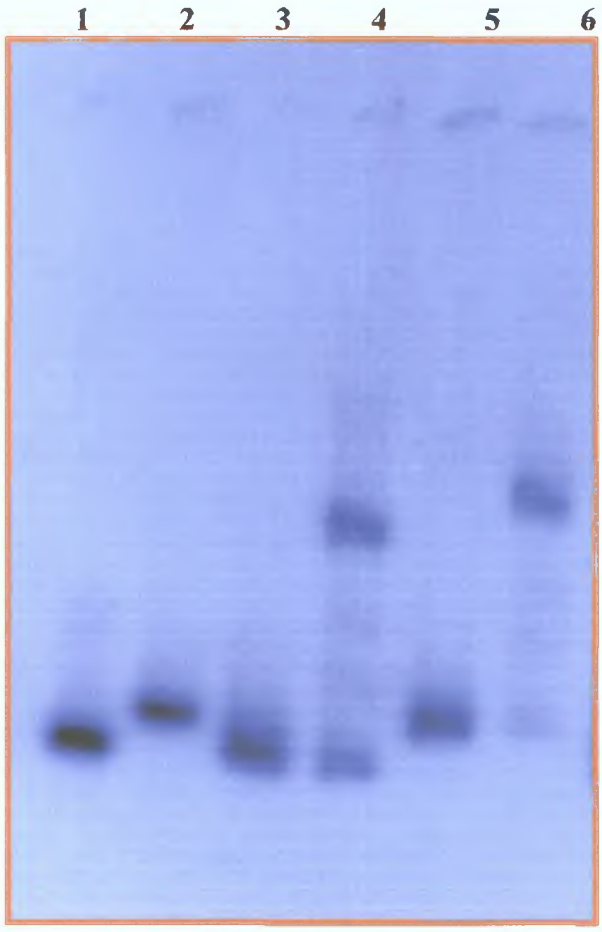


Fig 4.33: Mobility shift assay with extracts containing RhrA

Negative controls
Lane 1: Probe 1 + Binding Buffer
Lane 2: Probe 2 + Binding Buffer
Probe 1: **Probe upstream *rhtx***
Lane 3: pQE60 IPTG induced + Binding Buffer + Probe
Lane 4: pRhrA60 IPTG induced + Binding Buffer + Probe
Probe 2: **Probe intergenic region *rhrA-rhtA***
Lane 5: pQE60 IPTG induced + Binding Buffer + Probe
Lane 6: pRhrA60 IPTG induced + Binding Buffer + Probe

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*rirA*2 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 *rhtXrhbABCDEF* upstream of a GFP reporter gene The different constructs were mobilised into the *S meliloti* 2011 wild type but also in 2011*rirA*2 When comparing the GFP activity emitted by pWT, carrying the intact promoter region, mobilised into 2011 and into 2011*rirA*2 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, analyse 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 m 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 *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-termmal This is, for example, the case for the regulatory functioning of FNR, the transcriptional regulator of anaerobic respiration of *E coli* Indeed, the interconversion of both forms of the protein appears to be regulated by the availability of O_2 but also by the binding of ferrous iron to the cysteine residues (Trageser *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 RirA is not restricted to the siderophore system suggests that RirA is a general iron regulator, like those proteins RirA also appears to be the general iron response regulator in *R leguminosarum* (Todd *et al* 2002) In contrast, in *B japonicum*, it has been found that an additional protein, Irr, functions along with Fur m the iron response (Hamza *et al* , 2000) There is no obvious reason why some rhizobia have recruited RirA as an alternative to Fur as the general iron response regulator

A parallel investigation was undertaken of 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 *rhtXrhbABCDEF* and in the intergenic region of *rhrA-rhtA* (Fig 4 34), two promoters known to be activated by RhrA (Lynch *et al* , 2001)

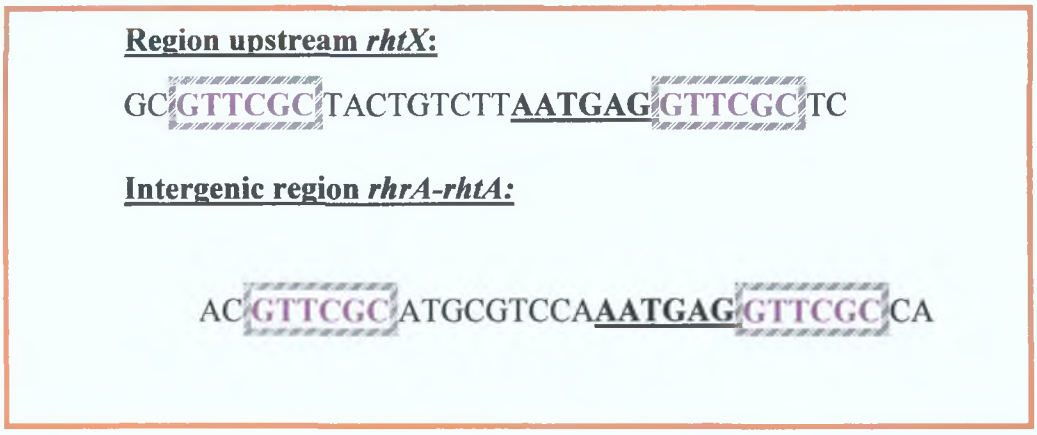


Fig 4.34: Region of the putative RhrA binding repeats upstream from *rhtX* and in the intergenic region of *rhrA-rhtA*.
The binding sites are framed and the 6-bp additional repeats between the two regions are underlined.

The results of the levels of GFP activity suggested that in *S. meliloti*, under iron deplete conditions, the expression of the operon *rhtXrhbABCDEF* 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 [pEN4] 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 *rhbA* 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-termmal 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 *rhtXrhbABCDEF* and *rhtA* provoking the positively regulation of those genes

Chapter 5:

Luteolin regulation of the siderophore biosynthesis gene *rhbG* 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).

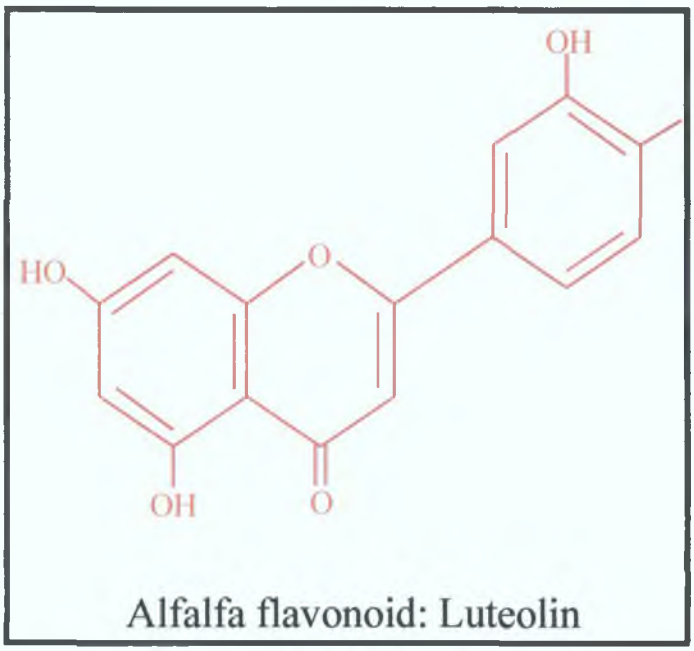


Fig 5.1: Chemical structure of luteolin, the inducer released from *M. truncatula*

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 macro-arrays 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 biomformatics 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 *in vivo* analysis of the luteolin regulation of *rhbG* under iron deplete and replete conditions.

An *rhbG*-Tn5*lacZ* mutant strain was previously generated in our laboratory by transposon insertion (Lynch, PhD thesis, 1999) and called 2011*rhbG*25 This strain carries the Tn5 *lacZ* transposon inserted in the chromosomal copy of the *rhbG* gene in the correct orientation The mutant was constructed using *S meliloti* G212, deletion mutant of *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 *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* G212*rhbG*25 under iron deplete and replete conditions and in the absence and presence of the inducer, luteolin.

	β-galactosidase activity (Miller Unit)	Standard error
Iron replete conditions		
S. *meliloti* G212	3.3	+/- 3.3
S. *meliloti* G212*rhbG*25	11.61	+/- 1.41
Iron replete conditions with the addition of luteolin		
S. *meliloti* G212	1.81	+/- 0.10
S. *meliloti* G212*rhbG*25	253.50	+/- 2.73
Iron deplete conditions		
S. *meliloti* G212	3.50	+/- 0.69
S. *meliloti* G212*rhbG*25	381.69	+/- 93.90
Iron deplete conditions with the addition of luteolin		
S. meliloti G212	2.92	+/- 0.10
S. meliloti G212*rhbG*25	763.12	+/- 61.64

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* 2011*rhbG*25 under iron replete conditions but a 33-fold expression increase was seen under iron deplete conditions (Table 5.1). This implies that *rhbG* 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 2011*rhbG*25 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).

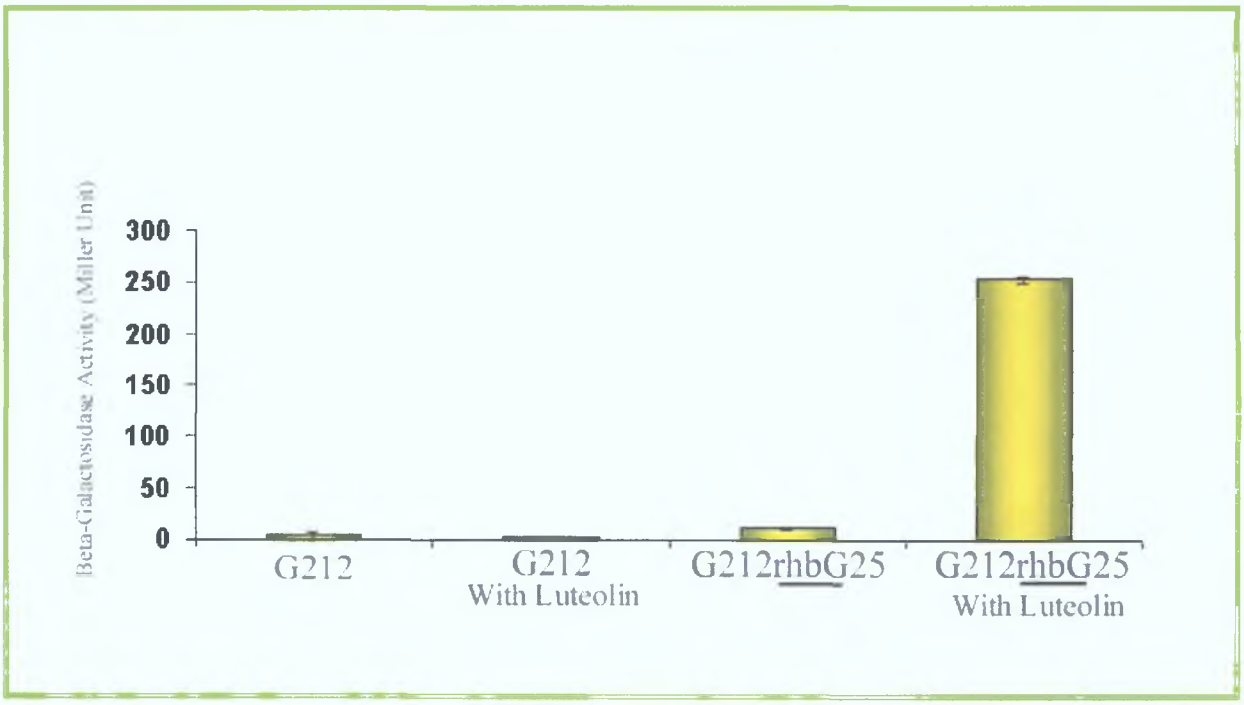


Fig 5.2: β-galactosidase activity (Miller U) under iron replete conditions in the presence and absence of luteolin of G212 and G212*rhbG*25.

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 *rhbG* 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 *rhbG.*

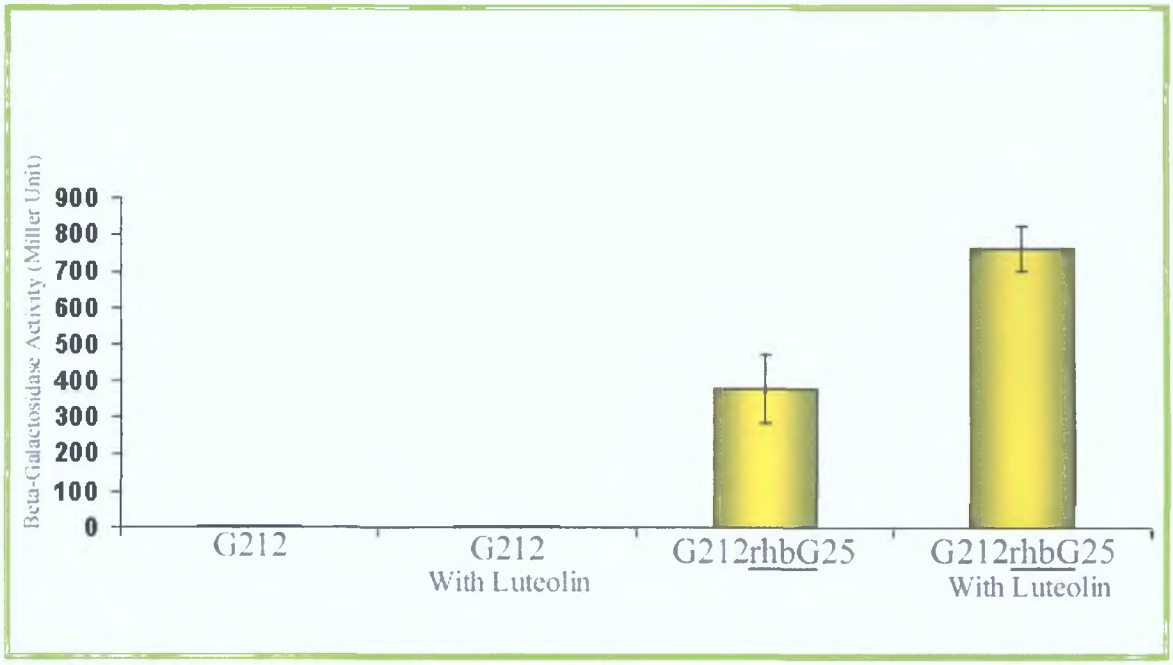


Fig 5.3: β-galactosidase activity (Miller U) under iron deplete condition in the presence and absence of luteolin of G212 and G212*rhbG*25.

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 teste 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 nitrogen-fixing ability in acetylene reduction was observed compared to the wild type (Lynch *et al*, 2001) Competition studies between *S meliloti* 2011 and 2011*rhbG*25 would allow an assessment of the importance of the lipid tail encoded by *rhbG*

Its 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 luteolm 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, daidzem 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 luteolm 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 luteolm 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 shows 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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