THE UNIVERSITY OF CALGARY

Organisation and Regulation of the *fixG* operon of *Rhizobium leguminosarum.*

by

Michael J. Mitsch

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE.

DEPARTMENT OF BIOLOGICAL SCIENCES

CALGARY, ALBERTA

SEPTEMBER, 1995

THE UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The organisation and regulation of the *fixG* operon of *Rhizobium leguminosarum*" submitted by Michael J. Mitsch in partial fulfilment of the requirements for the degree of Master of Science.

Supervisor, Dr. Michael F. Hynes, Dept. of Biological Sciences

Dr. G. Voordouw, Dept. of Biological Sciences

Dr. S. L. Wong, Dept. of Biological Sciences

External Examiner, Dr. Denis Gaudet, Research Scientist, Agriculture Canada

September 7, 1995.

Abstract

Utilising a probe derived from the *Rhizobium meliloti fixG* operon two homologous regions in *Rhizobium leguminosarum* biovar *viciae* strain VF39 were identified. The two copies are plasmid based, found on pRLeVF39c and pRLeVF39d and are located downstream of other *fix* genes forming two similar gene clusters characteristic of the *Rhizobium* genera. Both homologues were isolated, mapped and sequenced utilising nested deletions. Regulation studies utilising *lacZ* fusions indicate that these operons are expressed at a higher rate under microaerobic conditions. Double and single mutants were produced using interposons and a gene replacement system based on the *sacB* gene of *Bacillus subtilis*. These mutants were utilised in plant tests to evaluate their nitrogen fixation ability and these experiments indicated that both copies are functional but only one copy is necessary for nitrogen fixation.

Acknowledgements

I would like to thank all the people I had the privilege of working with while completing this work. Among them are my lab mates Dr. Jürgen Quandt, Dr. Rhonda Clark, Dr. Patrice Rochepeau, Laurie Faas, Chris Yost, Tanya Noel, and Sunny Twelker. They provided not only technical expertise but also moral support and a shoulder to cry on when I needed it. I would also like to thank my friends in the department; Danita, Tod, Julian, Kathy, Arin and especially my girl friend Alisa who filled my non-work hours with so much fun and laughter. I have been blessed with good friends. I also thank the members of my committee who generously gave their time to read and comment on this paper. I would also like to acknowledge the help and support of my family who gave me the opportunity to arrive at this point in my career with their guidance and financial generosity, I owe them a great deal. Finally I would like to thank Dr. Michael Hynes for all that he has done for me in the time that I have known him. He gave me the chance to work in a laboratory, attend graduate school, and learn a great deal about many things. I thank him for his encouragement, support, and a kick in the rear when I needed it. Thank you Sir.

Dedication

Dedicated to my co-workers, friends and family without whom I would not have been able to accomplish this task.

Table of Contents

Section	Page
Approval Page	ii
Abstract	iii
Acknowledgements	iv
Dedication	v
Table of contents	vi
List of Tables	viii
List of Figures	ix
Abbreviations and symbols	x
Chapter1: Introduction	1
1.1 Importance of Biological nitrogen fixation	1
1.2 Overview of Biological nitrogen fixation	2
1.3 *fix* genes; their organisation and activation in the nodule	4
1.4 *fix* gene activators	7
1.4.1 FixL structure and function	10
1.4.2 FixJ structure and function	11
1.4.3 Model of FixLJ activity	12
1.5 Targets of FixLJ activation	13
1.5.1 *nifA*	13
1.5.2 NifA structure	14
1.5.3 NifA function	15
1.5.4 *fixK*	16
1.5.5 FixK structure	16
1.5.6 FixK function	17
1.6 Operons required for symbiotic nitrogen fixation	18
1.6.1 *fixABCX*	18
1.6.2 *fixNOQP*	18
1.6.3 *fixGHIS*	20
1.7 Summary	20
1.8 Objectives	21

Chapter 2: Materials and methods 23
2.1 Bacterial strains and plasmids utilised 23
2.2 DNA manipulations 23
2.3 Southern hybridisations 28
2.4 Nested deletions and sequencing 28
2.5 *R. leguminosarum* manipulations 29
2.6 Beta-galactosidase assays 30
2.7 Plant tests 31

Chapter 3: Results and discussion 33
3.1 Identification of *fixGHIS* operons 33
3.2 Operon inactivation 33
3.3 Plant test results 42
3.4 Sequence comparisons of the *R. leguminosarum fixG* operon 54
3.5 Activational studies 68
3.5.1 Succinate induction 68
3.5.2 Microaerobic induction 75
3.5.3 Copper activational studies 79

Chapter 4: Conclusions 82

Chapter 5: References 85

Appendix 1: Medium and commonly used solutions 98

Appendix 2: Plasmids produced 101

List of Tables

Table		Page
Table 2.1	Strains and plasmids used	24
Table 3.1	Nitrogenase activity	52

List of Figures

Figure		Page
Figure 1.1	Nodule diagram	5
Figure 1.2	Location of nitrogen fixation genes	8
Figure 3.1	Identification of the two *fixG* operons	34
Figure 3.2	Cosmids containing *fixG* segments	36
Figure 3.3	Map of *fixG* operons	38
Figure 3.4	Operon inactivation	40
Figure 3.5	Gene replacement vector	43
Figure 3.6	Effects of mutants on plant hosts	45
Figure 3.7	Plant dry weights	47
Figure 3.8	Verification of plant test sterility	50
Figure 3.9	Protein comparison of the two operons	55
Figure 3.10	Nucleotide comparison	57
Figure 3.11	Interspecies protein comparison	61
Figure 3.12	Copper pump structural comparison	64
Figure 3.13	Promoter comparison	66
Figure 3.14	Reporter gene construct	69
Figure 3.15	Carbon source effects	72
Figure 3.16	Microaerobic effects	76
Figure 3.17	Copper effects	80

Abbreviations and symbols

ATP	Adenosine triphosphate
bp	Base pair
Crp	cAMP receptor protein
Dct^-	The inability to import carbon sources
DNA	Deoxyribonucleic acid
Fix^+	The ability to fix nitrogen
Fix^-	The inability to fix nitrogen
Fnr	Transcriptional regulator of anaerobic respiration
Gm	Gentamycin
kb	Kilobase
kDa	Kilodalton
IHF	Integration host factor
LB	Liquid broth
mRNA	Messenger RNA
Nif	Dysfunctional nitrogenase enzyme
Nm	Neomycin
nm	Nanomolar
PA	Antibiotic broth
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SSC	Tri sodium citrate
Sm	Streptomycin
Sp	Spectinomycin
TB	Terrific broth
TY	Tryptone yeast
UAS	Upstream activator sequence
VMM	Vincents minimal media

Chapter 1. Introduction

1.1 Importance of biological nitrogen fixation

In the third century B.C. the first recorded observation of *Rhizobium* assisted plant growth was made by Theophrastus (Raven *et al.* 1986) who described the use of faba beans to enrich the arable lands of Greece. In 1993 80 million tons of nitrogen based synthetic fertilizer was used at a cost in excess of 20 billion dollars to maintain the high yields of modern crop cultivars to feed the worlds population (Hardy, 1993). This reliance on chemical fertilizers is costly, not only in financial terms, because their production requires the burning of non-renewable fossil fuels. Furthermore less than half of the nitrogen is assimilated by the targeted plants while the remainder is metabolised by soil organisms to form other compounds which can contaminate ground water or produce the greenhouse gas nitrous oxide (National Research Council, 1994). Alternatively, biological nitrogen fixation produces approximately 100-175 million tons of nitrogen annually (Hardy, 1993) with 60% of this volume in conjunction with agricultural crops (Bezdicek *et al.* 1988) and most of it being assimilated by the plant host. With the growing concern of the world's expanding population and the environmental implications associated with synthetic chemical fertilizers, it has become imperative to take full advantage of bacterial-plant associations and symbiotic nitrogen fixation. For these reasons, a great deal of research has been undertaken with the members of the rhizobia which include *Rhizobium leguminosarum,* the symbiont of peas (*Pisum sp.*), lentils (*Lens sp.*), and clover (*Trifolium sp.*), *Rhizobium meliloti* which nodulates alfalfa (*Medicago sativa*) and sweet clover (*Melilotus sp.*), *Bradyrhizobium japonicum* which fixes nitrogen in association with soybeans (*Glycine max*) and other tropical plants, and *Azorhizobium caulinodans* which can fix nitrogen in both a free living state and in conjunction with the tropical legume *Sesbania rostrata* (Pawlowski *et al.* 1987).

1.2 Overview of biological nitrogen fixation

In 1965, the ability to fix atmospheric nitrogen was demonstrated in the free living bacterium *Klebsiella pneumoniae* (Mahl *et al.* 1965). From these initial observations research has been conducted to locate and characterise the nitrogen fixation or *nif* genes responsible for the entire process. These studies have shown that the genes responsible for nitrogen fixation are grouped together on a 25 kb region of the bacterial chromosome and are made up of more than 20 separate coding regions. Through genetic analysis and physical characterisation of mutants unable to fix nitrogen (Nif^-), cloned fragments, and sequencing data, the function and necessity of each gene has been elucidated (Cannon *et al.* 1985, Merrick, 1993). The genes can be broken down into four groups based on related function. The first group is composed of the *nifK, nifD,* and *nifH,* genes which code for the structural elements of the nitrogenase enzyme. They create the dinitrogenase reductase Fe protein and the dinitrogenase FeMo protein which reduces N_2 to ammonium. The nitrogenase enzyme requires a specific electron transport chain to function and this is coded for by the second group of *nif* genes *nifJ* and *nifF*. The products of these genes have been purified and both appear to be flavodoxins and mediate electron transfer to the Fe protein of the nitrogenase enzyme (Shah *et al.* 1983). *nifJ* encodes a pyruvate:flavodoxin oxidoreductase which donates electrons to the flavodoxin protein NifF which passes on the electrons to the nitrogenase enzyme (Merrick, 1993). The third group of genes is made up of *nif Q,B,N,E,V,* and *S* which code for the biosynthesis of the FeMo cofactor, homocitrate synthase and cysteine-dependent S transferase for FeS clusters (Merrick, 1993). The fourth and final group of *nif* genes are the regulatory elements of the entire process. The products of the genes *nifA* and *nifL* are, respectively, activators and inhibitors of *nif* gene expression at the level of transcription. These regulatory elements are in turn controlled by the presence of oxygen or ammonium in the cell which is detected by the nitrogen regulation (NTR) system. Under low ammonium concentrations NtrB phosphorylates NtrC which, in

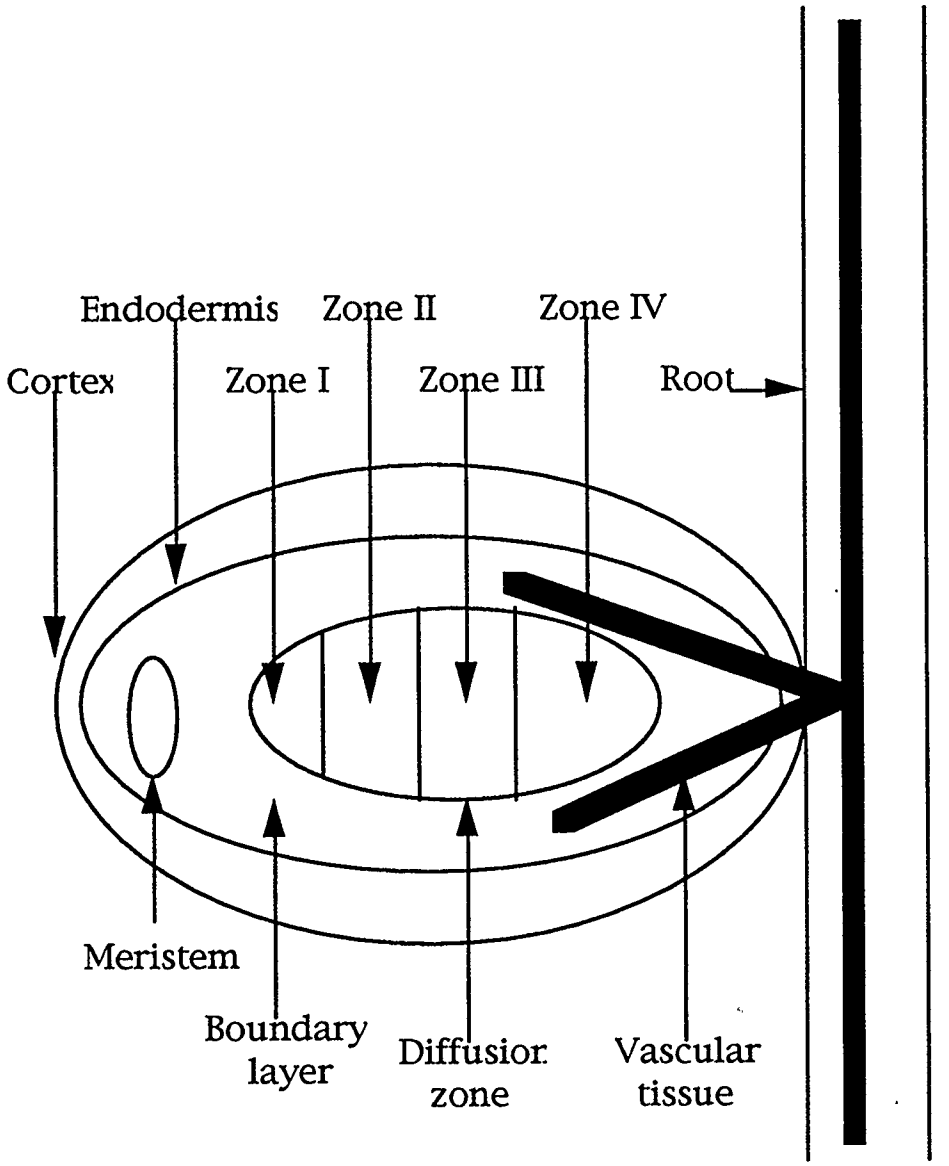
conjunction with NtrA, acts as a transcriptional activator of *nifA* and *nifL* genes which form the elements necessary for nitrogen fixation to be activated. Down regulation of this system depends on the fixed nitrogen sensors GlnD and GlnB. When intracellular nitrogen levels are high the ratio of glutamine to 2-ketoglutarate is also high. This is detected by GlnD, a uridylyltransferase. GlnD deuridylyates GlnB-UMP exposing the protein's phosphatase ability. The activated GlnB protein dephosphorylates NtrC and the protein is no longer able to act as a transcriptional activator. Thus *nifA* expression is curtailed and the nitrogen fixing proteins are not produced (de Bruijin *et al.* 1990).

The genes responsible for symbiotic association and nitrogen fixation are found on the large stable plasmids characteristic of symbiotic nitrogen fixers and can be separated into three groups with each of the groups responsible for a single aspect of the plant-microbe association. The bacterial *nod* genes are induced when flavonoids or isoflavonoids are released from the plant host, inducing the bacteria to release nod factors which enhance the curling of the plant root hairs (Dénarié *et al.* 1992). The bacteria then move towards the root and induce the formation of an infection thread by the host which they then use to penetrate the plant tissue. Once inside, the bacteria differentiate to form bacteroids and the *nif* genes are activated. These genes, which are highly homologous to the nitrogen fixation genes of the free living *K. pneumoniae* (Cannon *et al.* 1985, Merrick, 1993) form the oxygen sensitive nitrogenase enzyme which begins fixing atmospheric nitrogen to ammonium for assimilation into the plant host (Ludden, 1993). The third set of genes are known as *fix* genes and were thought, until recently, to be unique to the symbiotic nitrogen fixers. They are required for symbiotic association and serve a dual role as they form both regulatory elements (Hennecke, 1990, Merrick, 1993, Monson *et al.*, 1993, Fischer *et al.* 1993) and structures (Arigoni *et al.* 1991, Renalier, 1987, David, 1987) required for nitrogen fixation *in planta.*

These unique genes and proteins may reflect the fact that symbiotic nitrogen fixers face different conditions and environmental challenges compared to their free living counterparts. These bacteria go from an aerobic (250 micromolar O_2) soil dwelling existence to a microaerobic environment (3-30 nanomolar O_2) (Hennecke, 1993) where carbon sources such as malate and succinate as well as oxygen via leghemoglobin are provided to them in exchange for fixed nitrogen. This change in environment coincides with drastic changes in the bacterium, known after differentiation and introduction into plant cells as a bacteroid. Originally seen as morphological alterations in size and shape (Hirsch and Smith, 1987) these cellular alterations are now known to involve changes in nucleic acid content, gene expression, cytoplasmic alterations, and cell membrane changes which occur due to environmental cues (Vasse *et al.* 1990). These cellular alterations occur at different locations within the developing nodule and Vasse *et al.* (1990) have proposed a nomenclature to describe these locations (see figure 1.1). The nodule itself consists of four distinct zones surrounded by plant tissue. Distal to the root is a bacteria-free meristimatic area, zone I. The bacteria enter the invasion or early symbiotic area, zone II which is separated from the third zone by a layer of amyloplast-rich cells. This area separating zone II and III is the location of activation of the *fix* and *nif* genes (de Maagd *et al.* 1994, Labes *et al.* 1993). Nitrogen fixation occurs in the microaerobic late symbiotic zone, zone III, and the fourth zone is made up of a senescence zone proximal to the root. Throughout this differentiation the bacteria are provided with carbon sources from the plant host including succinate, malate, and fumarate which are imported via a specialised dicarboxylate transport system known as the Dct system and coded for by *dctA, dctB, and dctD.*

1.3 *fix* genes; their organisation and activation in the nodule

Figure 1.1. Diagrammatic representation of a nodule showing plant structures and zones of bacteroid differentiation. Modified from Werner (1992) and Vasse *et al.* (1990).

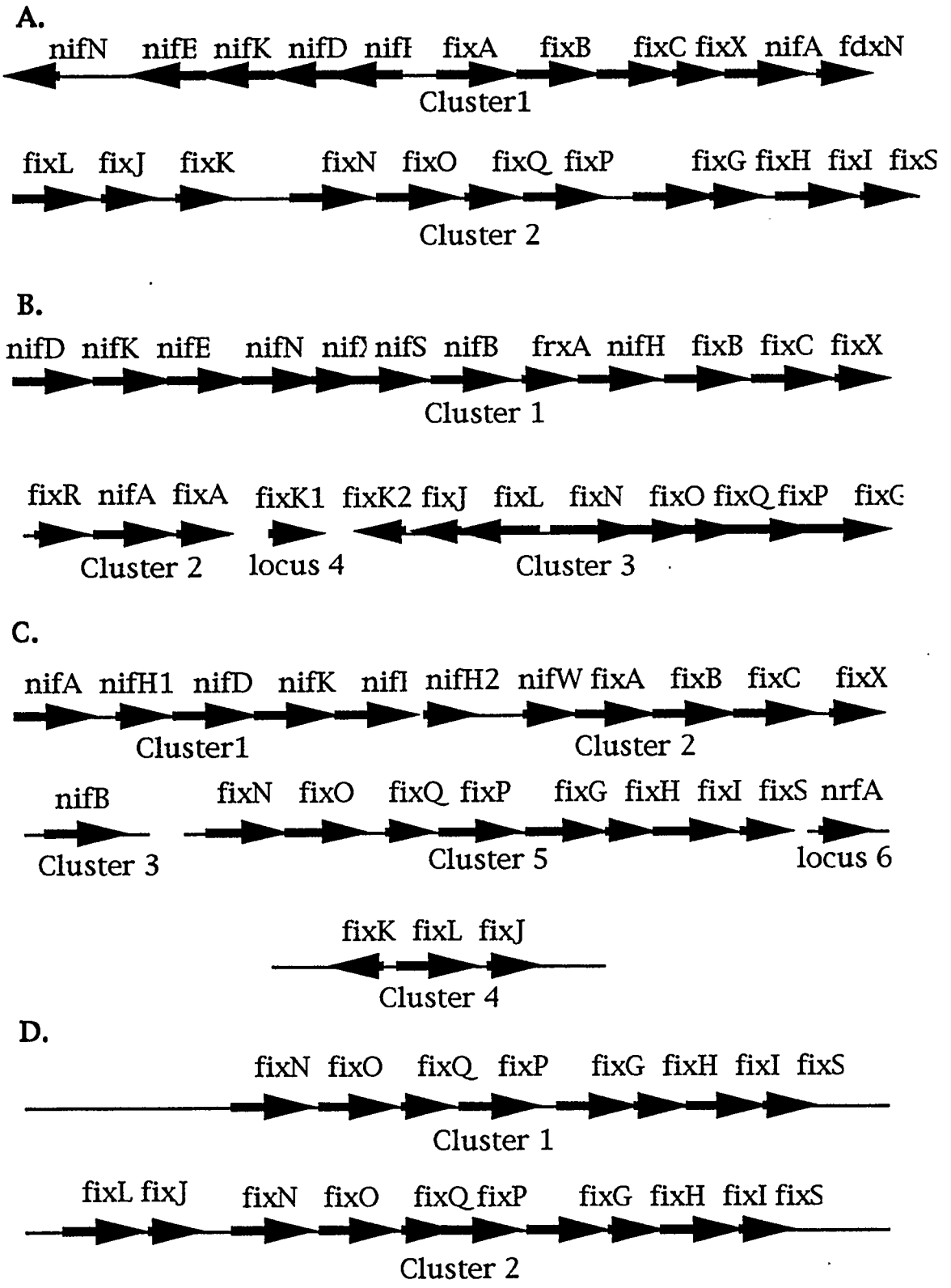

Endodermis
Zone II
Zone IV
Cortex
Zone I
Zone III
Root
Meristem
Boundary layer
Diffusion zone
Vascular tissue

In order to fix atmospheric nitrogen, oxygen concentrations within the nodule must be lowered in order for the nitrogenase enzyme to function. This creation of a microaerobic environment where free oxygen concentrations can be as low as 3 to 30 nM (Hennecke, 1993) activates several *fix* genes and gene products which control the nitrogen fixation pathway (Ditta *et al.* 1987). In Rhizobium species these genes are located on the large, stable plasmids characteristic of *R. meliloti* (Renalier *et al.* 1987) and *R leguminosarum* (Hynes and McGregor, 1990, Innes *et al.* 1988). In *R. meliloti* the two *fix* clusters essential for nitrogen fixation are located on the pSym plasmid while in *R. leguminosarum* two *fix* clusters have been found on the C and D plasmids of strain VF39 (Hynes *et al.* unpublished data). In *B. japonicum* and *A. caulinodans* the chromosomally based *fix* genes have a similar organisation; two clusters have been discovered in *B. japonicum* (Hennecke, 1990, Hennecke *et al.* 1985) while in *A. caulinodans* four clusters have been isolated as well as two other loci coding for single nitrogen fixation genes (Donald *et al.*, 1986, Pawlowski *et al* 1989) (see figure 1.2). In all of the rhizobia these *fix* clusters are activated by low oxygen conditions created within the nodule. The membrane bound oxygen sensing proteins work as a two component regulatory element much like the *ntrB* and *ntrC* nitrogen assimilation system (Nixon *et al.* 1986) where one protein acts as a membrane bound receptor while the second protein acts as a transcriptional activator of certain genes. Extensive research has been conducted into the regulatory elements of the nitrogen fixing symbionts and the FixL and FixJ two step regulatory components have been identified along with the *fix* gene transcriptional activating protein FixK.

1.4 *fix* gene activators

fixLJ were first identified in *R. meliloti* based on their necessity for activation of the *nifA* gene under microaerobic conditions (David *et al.* 1988) as well as the *fixNOQP* cluster required for symbiotic nitrogen fixation (David *et al.* 1987). These initial studies prompted further research into other members of the

Figure 1.2. Diagrammatic representation of the nitrogen fixation genes in *R. meliloti* (**A**), *B. japonicum* (**B**), *A. caulinodans* (**C**), and *R. leguminosarum* (**D**). Modified from Fischer (1994).


A.
nifN nifE nifK nifD nifI fixA fixB fixC fixX nifA fdxN
Cluster1
fixL fixJ fixK fixN fixO fixQ fixP fixG fixH fixI fixS
Cluster 2
B.
nifD nifK nifE nifN nifJ nifS nifB frxA nifH fixB fixC fixX
Cluster 1
fixR nifA fixA fixK1 fixK2 fixJ fixL fixN fixO fixQ fixP fixG
Cluster 2
locus 4
Cluster 3
C.
nifA nifH1 nifD nifK nifI nifH2 nifW fixA fixB fixC fixX
Cluster1
Cluster 2
nifB
fixN fixO fixQ fixP fixG fixH fixI fixS nrfA
Cluster 3
Cluster 5
locus 6
fixK fixL fixJ
Cluster 4
D.
fixN fixO fixQ fixP fixG fixH fixI fixS
Cluster 1
fixL fixJ fixN fixO fixQ fixP fixG fixH fixI fixS
Cluster 2

rhizobia and homologs were found in *B. japonicum* (Anthamatten and Hennecke, 1991), *A. caulinodans* (Kaminski and Elmerich, 1991), and *R leguminosarum* (Hynes *et al.* 1992). Based on sequence data and protein purification FixL was found to be a membrane-bound 55kDa protein while FixJ is a cytoplasmic DNA binding protein approximately 22kDa in size.

1.4.1 FixL structure and function

FixL in *R. meliloti* was found to be a membrane bound oxygen sensor that controlled the expression of two genes, the transcriptional activators *fixK* and *nifA* via FixJ (de Philip *et al.* 1990). A truncated FixL protein lacking 126 amino acids of the N-terminus was produced by Gilles-Gonzalez *et al.* (1991) and this soluble protein (known as FixL*) was used to demonstrate that FixL was a hemoprotein with kinase activity and is therefore responsible for the transfer of the gamma phosphate of ATP to the FixJ protein. FixL was dissected further and it was found that amino acid residues 127 to 260 are required to bind heme and oxygen but lack the kinase ability which was localised to the C-terminus, beginning after residue 260 (Monson *et al.* 1992). Further localisation of active sites has revealed that mutations of histidine residue 194 inhibit heme binding, and abolish the ability of FixL to control its phosphorylation/dephosphorylation activity in the presence of oxygen, while mutation of histidine 285 causes the loss of autophosphorylation activity (Monson *et al.* 1995). Tn*phoA* insertional analysis was carried out, and this identified the structural motif of the N-terminus as being that of a polytopic integral membrane protein with four membrane spanning segments that is not required for oxygen sensitivity (Lois *et al.* 1993a). FixL also undergoes a spontaneous autophosphorylation in the presence of ATP and this phenomenon is accelerated in the absence of oxygen while the transfer of the gamma phosphate is independent of oxygen concentrations (Gilles-Gonzalez and Gonzalez, 1993). In addition to the kinase activity, it is now clear that FixL contains a phosphatase activity that is repressed under anaerobic conditions

when the protein is phosphorylated (Lois *et al.* 1993b) and this ability may represent a fine tuning mechanism for the regulatory process. It has also been hypothesised that FixL may play a role in the cellular response to ammonia. *nifA* expression appears to be down regulated in the presence of ammonia and this response is not mediated via FixK (Noonan *et al.* 1992), another *nifA* activator leaving the FixLJ system as a prime candidate however no verification of this work has been conducted.

Like *R. meliloti*, *B. japonicum* and *A. caulinodans* also contain *fixLJ* genes. They are 50% identical to their *R. meliloti* counterpart and any mutation causes a severe curtailing of nitrogenase activity. However they play no role in the expression of *nifA* and instead appear to control other genes required for symbiotic nitrogen fixation and microaerobic respiration (Anthamatten *et al.* 1992). Alternatively, in *R. leguminosarum* a *fixLJ* homolog has been identified on the plasmid pRleVF39c and seems to affect the expression of *fix* operons (Hynes *et al.* 1992).

1.4.2 FixJ structure and function

FixJ was identified along with FixL and was found to be made up of 204 amino acids and to share homology with the OmpR protein of *E. coli*, a regulatory element in the production of outer membrane proteins for osmotic regulation (David *et al.* 1988). The N-terminus of this gene resembled several regulatory proteins while its C-terminus was homologous to the transcriptional activator UhpA of *E. coli*. With this information the connection was made that FixJ was a regulatory element that was activated by FixL and goes on to control other genes as a transcriptional activator (David *et al.* 1988). The hypothesis was tested in an *E. coli* background using an overexpressed *fixJ* and *pnifA:lacZ* and *pfixK:lacZ* fusions. It was found that overexpression of FixJ allows it to function in the absence of FixL and this may be due to "crosstalk" with similar phosphorylation proteins within *E. coli*. The most important result of these experiments was the identification of FixJ's target genes, *fixK* and *nifA*, the two activator elements

required for nitrogen fixation to occur (Hertig *et al.* 1989). The sigma factor required for proper functioning of the FixJ-*pfixK* interaction was elucidated by Batut and associates (1991) and was found to depend on the sigma 70 holoenzyme form of RNA polymerase. Structural motifs of FixJ were also examined to isolate the amino acids required for the FixJ functions. The C-terminus of FixJ was found to house the DNA binding elements of the protein as it has the characteristic helix-turn-helix motif indicative of all transcriptional activators and also contains an area of homology with region 4 of different sigma factors which is needed for the identification of -35 sequences in promoters (Kahn and Ditta, 1991). The N-terminus was found to contain the phosphorylation site that allows activation of the protein once FixL has donated the gamma phosphate and that Asp 54 is a vital component for the acceptance of this phosphate (Reyrat *et al.* 1994). These results were supported by Weinstein who used mutant FixJ proteins to show that phosphorylation is necessary for activation of the DNA binding and transcriptional events to occur (Weinstein *et al.* 1992). To prove the notion that oxygen regulates the activity of FixJ via FixL, studies were carried out using a soluble form of FixL (FixL122). It was found that FixJ phosphorylation was enhanced under low oxygen concentrations and that this resulted in increased activity of *nifA* and *fixK* transcription (Reyrat *et al.* 1993). The situation appears to be similar in *B. japonicum* as its FixJ protein is 205 amino acids in length and shares close to 50% identity to its *R. meliloti* counterpart and also acts as a transcriptional activator of *fixK* (Anthamatten and Hennecke, 1991, Anthamatten *et al.* 1992). In *R. leguminosarum* a *fixJ* homolog has been identified on the C plasmid but its role in *fix* gene expression is unknown (Priefer, personal communication, Hynes *et al.* 1992). For *A. caulinodans* the protein is 211 amino acids in length but shares the same structures and function as the other homologs.

1.4.3 Model of FixLJ activity

Based on the above information, a model for FixLJ activity can be formulated. Low oxygen concentrations are detected by the heme moiety of the membrane bound FixL, and this causes the activation of the C-terminus kinase and a curtailing of the phosphatase abilities of the protein. Once phosphorylated, FixL transfers the phosphate group to the N-terminus of FixJ which causes the exposure or activation of the C-terminal DNA binding domain which recognises certain promoter structures important for nitrogen fixation.

1.5 Targets of FixLJ activation

The transcriptional activator FixJ has two targets in *R. meliloti*; *nifA* and *fixK*. The proteins produced by these two genes act as activators for the entire nitrogen fixation pathway with genes activated by NifA responsible for the formation of the nitrogenase enzyme and associated electron transport pathways while FixK activates the operons responsible for the formation of symbiotic specific components. The two genes share similar promoter motifs in the different rhizobium species with a conserved 5'-C(C/G)NAAT(T/A)T-3' located 33bp upstream from the transcriptional start site and a unique 5'-TAAG-3' element 30 bp upstream from the first motif which is limited to *fixK* genes (Agron *et al.* 1992, Waelkens *et al.* 1992, Agron *et al.* 1993).

1.5.1 *nifA*

As with most elements in the nitrogen fixation pathway, *nifA* and its associated gene product were first found in *K. pneumoniae* (Buchanan-Wollaston *et al.* 1981). Once isolated, this clone was then used as a probe and several *nifA* homologs were identified in different nitrogen fixing bacteria including *R. meliloti* (Szeto *et al.* 1984), *R. leguminosarum* (Grönger *et al.* 1987, Watson *et al.* 1988) *B. japonicum* (Thöny *et al.* 1987), and *A. caulinodans* (Nees *et al.* 1988). In *R. meliloti* the *nifA* gene was originally identified by Tn5 mutagenesis to regions in and around the nitrogenase genes but its function and location were not pinpointed (Ruvkun *et al,* 1982)

until plant tests conducted with these mutants found that a region 5kb upstream of the *nif* genes results in the loss of the nitrogenase enzyme and a resulting fix$^-$ phenotype (Zimmerman *et al.* 1983). The mutation responsible for the absence of the nitrogenase enzyme was traced to a 1.8kb region 5.5kb upstream from the *nif* cluster and was found to activate a *nifH-lacZ* fusion and also hybridised to the *nifA* gene isolated from *K. pneumoniae* (Szeto, *et al.* 1984). The *nifA* gene was also identified in *B. japonicum* and through sequence and hybridisation data was shown to be similar to its *K. pneumoniae* counterpart and capable of activating *nifH:lacZ* fusions (Fischer, *et al.* 1988). Similarly in *A. caulinodans* the *nifA* gene was isolated and sequenced and was found to share a great deal of homology with *nifA* genes in other rhizobium species as well as *K. pneumoniae* (Ratet *et al.* 1989). The *nifA* gene of *R. leguminosarum* was found to reside between *nifB* and *fixX* and was required for nitrogenase expression and symbiotic nitrogen fixation (Iismaa and Watson, 1989, Grönger *et al.* 1987).

1.5.2 NifA structure

In terms of structure, all the NifA proteins are close in size ranging from 519-626 amino acids. They have several domains with variable homology, each responsible for a separate function. The N-terminus ranges from 216 amino acids in *A. caulinodans* to 164 amino acids in *R. meliloti* and shows a variable structure with only 29-46% identity within this region. The function of the N-terminus remains unknown and appears not to serve an important role as deletion of this area does not impair *nif* gene activation (Beynon *et al.* 1988, Huala *et al.* 1989, Fischer *et al.* 1988). In fact in *R. leguminosarum* the predicted protein sequence lacks the N-terminal domain seen in other rhizobia (Iismaa and Watson, 1989). It is possible that the N-terminus acts as stabiliser of NifA much as eukaryotic proteins are stabilised by the amino end of the protein. The N-terminus is separated from the rest of the protein by a short Q-linker region which is glutamine rich and hydrophobic in nature. These regions are common in modular proteins and serve to

separate functional domains (Wootton and Drummond, 1989). The central domain shows a high degree of similarity between the rhizobium species with between 53-72% identical amino acids being present in the roughly 240 residue region. It has been shown in *R. meliloti* that this region is essential for transcriptional activation of genes under NifA control, and that it may be responsible for interactions with the transcriptional machinery such as sigma 54 to initiate transcription (Huala and Ausubel, 1989). A similar system exists in *B. japonicum* (Fischer *et al.* 1988) and *A. caulinodans* (Ratet *et al.* 1989). A second linker connects the central activator domain to the C-terminal domain in rhizobium species. This linker region is made up of between 32-44 amino acids and contains a conserved Cys-X11-Cys-X19-Cys-X4-Cys sequence which resembles the protein binding motif of some metal cofactors. Experiments with *B. japonicum* have found that elimination of this region or manipulation of the distance between the two closest cysteine residues results in loss of NifA activity (Fischer *et al.* 1988). It has been proposed that the cysteine residues co-ordinate a metal cofactor which is required for NifA activity via its redox state (Morett *et al.* 1991). The C-terminus of NifA contains a helix-turn-helix motif with variable length in the different rhizobial members (67, 78, and 86 amino acids in *R. meliloti, B. japonicum,* and *A. caulinodans* respectively) and between 41 to 56% identical amino acids. The structure of this region combined with the fact that the same region in *K. pneumoniae* is capable of binding to UAS sites of *nif* genes (Morett and Buck, 1988) has led to the conclusion that the C-terminus of NifA is responsible for DNA binding via the second helix which shows specificity for NifA regulated genes (Morret *et al.* 1991)

1.5.3 NifA function

Once activated by the FixLJ system, NifA activates the genes responsible for the formation of the nitrogenase enzyme (*nif HDKE*), the FeMo cofactor (*nifN*), electron transfer proteins (*fdxN*), as well as the genes responsible for the formation of a putative electron

transport chain (*fixABCX)* for the nitrogenase enzyme. NifA utilises its C-terminal domain to bind an UAS with the consensus sequence 5'-TGT-N_{10}-ACA-3' which is present at most NifA dependent genes 80 to 150 bp upstream from the transcriptional start site (Morett and Buck, 1988, Morett *et al.* 1991). Once bound, NifA comes into contact with the sigma 54-RNA polymerase located at the promoter region of the gene via loop formation facilitated by IHF located between the UAS and the promoter (Santero *et al.* 1989). Once NifA and the holoenzyme are in close association, the central domain assists in the initiation of transcription and the mRNA is formed.

1.5.4 *fixK*

fixK was originally identified in *R. meliloti* by *lacZ* fusions in the *fixN* operon which required the presence of a second regulatory element linked to *fixLJ* in the *fix* cluster for expression (Batut, *et al.* 1989). Similar genes were then found in *R. leguminosarum* (Colonna-Romano *et al.* 1990, Priefer, personal communication), *A. caulinodans* (Kaminski *et al. 1991*) and *B. japonicum* (Anthamatten *et al.* 1992). Interestingly *B. japonicum* seems to contain two homologs of the *fixK* gene but only one of these ($FixK_2$) is under the control of the FixLJ process and has any role in nitrogen fixation while the reiterated copy ($FixK_1$) has no identifiable function as yet. The proteins themselves are very similar and share a great deal of homology with the Crp-Fnr protein group. This family of regulatory proteins activate genes required for catabolic functions and anaerobic respiration by binding DNA and activating transcription (Kolb *et al.* 1993). With this information, along with activation studies using *lacZ* fusions and transposon mutagenesis it has been concluded that FixK serves much the same role in the rhizobium species and activates the *fix* genes required for symbiotic nitrogen fixation.

1.5.5 FixK structure

The four FixK proteins found in the rhizobium genera are similar in size containing 211 to 248 amino acids but beyond that

their structure is quite variable. FixK1 of *B. japonicum* and the FixK homolog in *R. leguminosarum* (FnrN) have four cysteine residues in the amino terminus of the protein which may be responsible for metal binding and/or redox sensing. This unique structure which is dissimilar from the other FixK proteins coupled with the fact that these two proteins have no elucidated function leads to the conclusion that they may act as secondary units for oxygen sensing and transcriptional activation (Schlüter *et al.* 1992) or as a substitute for the actual FixK element (Anthamatten *et al.* 1992). Alternatively, they may represent the classical Fnr proteins as they can replace Fnr *in vivo* and this may indicate that the remaining FixK homologs have evolved as strict regulators of *fix* operons required for nitrogen fixation *in planta.* All the FixK homologs contain a glycine rich area near the amino terminus characteristic of Crp-Fnr related proteins and a helix-turn-helix motif in the C-terminus which is indicative of a DNA binding domain.

1.5.6 FixK function

Once expressed in the rhizobium species, FixK universally activates the *fixNOQP* operon and other operons which are required for symbiotic nitrogen fixation. However, the activity on *nifA* expression varies in each organism. In *R. meliloti* FixK acts as a repressor of *nifA* activity as well as *fixK* transcription and therefore limits the production of the nitrogenase enzyme and other complexes required for nitrogen fixation (Batut *et al.* 1989). In *R. leguminosarum* no connection has been made with FnrN and *nifA* expression nor has any effect been seen in *B. japonicum* except that FixK2 plays a role in oxygen control of *fixK*1 and limits the activity of *fixK*2 (Fischer, 1994). At the other extreme, *A. caulinodans* FixK is necessary for *nifA* activation and transcription (Kaminski *et al.* 1991). The promoter elements required for FixK binding and activation have been elucidated and a consensus sequence of 5'-TTGA-C--GATCAA-G-3' has been identified. In *R. meliloti* two of these elements, known as anaeroboxes were found at 43bp and at 487bp upstream from the transcriptional start site of *fixK*. *B.*

japonicum also contains similar structural motifs in the promoter region of *fixK*$_2$ and these facts point to a DNA looping mechanism for down regulation of these genes. This motif has also been found within the promoter regions of several *fix* genes of different rhizobium species including *fixN* (Batut *et al.* 1989, Hynes *et al.* 1992, Preisig *et al.* 1993, Mandon *et al.* 1994) and *fixG* (Kahn *et al.* 1989, Preisig *et al.* 1993).

1.6 Operons required for symbiotic nitrogen fixation

Once induced by low oxygen concentrations, the regulatory factors of the rhizobium species activate the genes which form the proteins required for symbiotic nitrogen fixation. These include the genes *nifHDKE* but also the *fix* genes which form complexes and structures necessary for symbiotic nitrogen fixation. So far, three operons have been characterised: *fixABCX, fixNOQP* and *fixGHIS.*

1.6.1 *fixABCX*

The *fixABCX* operon was first found in *R. meliloti* (Corbin *et al.* 1983, Earl *et al.* 1987) but has now been identified in *B. japonicum* (Fuhrmann *et al.* 1985), *A. caulinodans* (Kaminski *et al.* 1988), *Azotobacter sp.* and several *R. leguminosarum* strains (Grönger *et al.* 1987, Iismaa *et al.* 1989). Any manipulation of this operon results in a loss on nitrogen fixation indicating that the protein products of these genes are essential for nitrogenase function. Sequence data of *fixX* indicates that it contains five conserved cysteine residues forming a cluster typical of ferredoxin proteins while *fixB* is similar to alpha subunits of eukaryotic electron transfer flavoproteins (Arigoni *et al.* 1991) and *fixC* has some homology to the human electron transfer flavo protein-ubiquinone oxidoreductase (Fischer, 1994). These observations coupled with the elimination of nitrogen fixation upon inactivation of these genes gave rise to the proposal that FixABCX may be an electron transport chain used to deliver electrons to the nitrogenase enzyme (Kaminski *et al.* 1991).

1.6.2 *fixNOQP*

The *fixNOQP* operon was first identified in *R. meliloti* (David *et al.* 1987) through transcriptional mapping and found to be a reiterated operon linked to the *fixLJK* regulatory genes. The operon was also discovered in *B. japonicum* (Preisig *et al.* 1993), *A. caulinodans* (Mandon *et al.* 1994), and a reiterated set of genes found in *R. leguminosarum* (Hynes *et al.* 1992, Quandt and Hynes, 1993, Schlüter *et al.* 1992). The free living nitrogen fixer *R. capsulatus* has a similar operon used for aerobic respiration known as *ccoNOQP* (Thöny-Meyer *et al.* 1994) while the plant pathogen *Agrobacterium tumefaciens* also contains a homologous oxidase for microaerobic respiration (Schlüter *et al.* 1995). The operon in all three rhizobium species was found to be regulated by FixK. Mutational studies carried out in *R. meliloti* and *B. japonicum* indicated that the operon is essential for symbiotic nitrogen fixation as its abolishment results in a Fix- phenotype. For *B. japonicum* *fixN-* mutants have a reduced oxidase activity under microaerobic and anaerobic conditions (Presig *et al.* 1993). In comparison, a *fixNO* mutant of *A. caulinodans* retained 50% of its nitrogenase activity in the symbiotic and free living state (Mandon *et al.* 1994). The sequence data and predicted amino acid sequence for this operon in *B. japonicum* and *A. caulinodans* is very similar and shows that FixN codes for a 549 to 551 amino acid protein which contains four histidine residues and transmembrane domains similar to subunit I of c-type and o-type terminal oxidases. FixO is 244 to 246 amino acids in length and contains a transmembrane helix near its N-terminus as well as a heme c binding motif (Cys-Tyr-Leu-Cys-His) at position 68-72 and a Met-Pro group at 140-141. The 54 amino acid FixQ has no characteristic patterns in its structure but does have a membrane spanning domain. FixP contains within its 290 amino acids an N-terminal membrane binding domain and two heme binding motifs that are highly homologous and may represent a duplication of an ancestral cytochrome similar in nature to c552/553 cytochromes. With this information, it has been hypothesised that FixNOQP forms a

membrane bound heme/copper terminal oxidase complex composed of a b/copper-binding subunit and c-type cytochromes.

1.6.3 *fixGHIS*

The final *fix* component found to date in the rhizobial species is the *fixGHIS* operon. It was first identified in *R. meliloti* (Kahn *et al.* 1989) and later in *B. japonicum* (Preisig *et al.* 1993) *A. caulinodans* (Mandon *et al.* 1993), *R. leguminosarum* (Hynes, unpublished data) and in the free living nitrogen fixer *Rhodobacter capsulatas* (Neidle and Kaplan, 1992). In *R. meliloti* and *B. japonicum* any interruption of the *fixGHIS* operon abolishes nitrogen fixation while in *A. caulinodans* symbiotic nitrogen fixation and free living nitrogenase functioning is only mildly impaired. The elimination of *fixGHIS* in *R. capsulatas* has no effect on nitrogen fixation. In *R. meliloti fixG* codes for a protein 524 amino acids in size which contains transmembrane helices and two cysteine clusters much like those found in bacterial ferredoxins and other redox proteins. FixH is 167 amino acids in length and has no unique structures except for a transmembrane region. FixI on the other hand is a 757 amino acid protein which bears a great deal of homology to cation ATPases of the P type. The fourth member of the operon, the putative *fixS* has not been determined to produce a protein of any comparable structure and since no mutations in this site have been produced, its characteristics are unknown. Based upon the predicted structure of the FixGHI elements it has been proposed that these proteins form a membrane bound complex where the cation pump is coupled to a redox reaction and that this complex is required for the import or export of a specific cation required for symbiotic nitrogen fixation.

1.7 Summary

Based on all of this information a picture of symbiotic nitrogen fixation can be created. Once symbiotic association is achieved, low oxygen concentrations within the nodule activate several genes required for nitrogen fixation. The regulatory

elements FixLJ sense the depressed oxygen levels and activate the *fix* genes through FixK and NifA. Some of these activated genes are the structural elements similar to those found in *K. pneumoniae* while others are unique to the rhizobia. These unique products form pathways required to deliver electrons to the nitrogenase enzyme (*fixABCX*), form respiratory pathways needed to overcome the low oxygen concentrations within the nodule (*fixNOQP*), and produce pumps to import cations for the formation and maintenance of these pathways (*fixGHIS*). Together these subunits allow the symbiotic association to function but there are several questions yet to be answered. In *R. leguminosarum* there are several reiterated genes which may or may not be required for symbiotic nitrogen fixation. Among them are the *fixGHIS* genes which are found in the symbiotic nitrogen fixers. These genes have been detected in both free living and symbiotic nitrogen fixers but their necessity appears to be variable and their function has yet to be elucidated. Also, the regulatory elements responsible for controlling this operon have not been identified. For these reasons a study into these genes has been undertaken.

1.8 Objectives

The objectives of this work were threefold; first to determine if both copies of the *fixG* operon in *R. leguminosarum* are functional and required for the formation of a Fix^+ phenotype or whether a single copy is sufficient. Second, to identify the regulatory elements of the operon based on sequence data as well as the environmental factors controlling the operons expression, and finally to compare the sequence of these operons to similar genes found in other bacteria.

The two operons found on the C and D plasmid were subcloned, mapped with restriction endonucleases and orientation determined via Southern hybridisation's. This facilitated the manipulations of the genes for further testing and experimentation. In order to assess if both operons were functional, single and double mutants of the two operons were produced using omega

interposons. These mutations were then introduced into the genome of wild type VF39 strains by utilising a gene replacement vector and homologous recombination. Verification of operon inactivation was assessed by band size alterations observed via Southern blots using *R. leguminosarum* total DNA and a *fixG* probe. The single and double mutants were then used as inoculants with sterile pea seeds along with VF39 wild type and uninoculated controls and grown under nitrogen starved conditions. After a sufficient growth period the plants were harvested, the presence of nodules determined, and the effects of single and double mutations assessed by a series of tests including dry weights of plant matter and acetylene reduction assays on the root systems.

Fusions with the *fixG* promoter and a promoterless *lacZ* cassette were produced and beta-galactosidase assays were carried out under a variety of conditions to assess promoter activation. In conjunction with this, the promoter sequence of the operons was determined and compared to other nitrogen fixation genes in order to identify any common elements or motifs. The operons themselves were sequenced and compared to the database in order to determine the degree of similarity between *R. leguminosarum* *fixG* operons and other genes.

Chapter 2. Materials and Methods

2.1 Bacterial strains and plasmids utilised

Bacterial strains were grown in either LB, PA, or TB for *E. coli*, and TY, PH, or VMM for *R. leguminosarum* VF39 strains (see appendix 1). All components used to produce bacterial media (LB, TY, PA, and PH) were purchased from BDH except for peptone which was obtained from Difco. Agar used for solid media production was obtained from Merck. Antibiotics for selection and screening were purchased from Sigma Chemical Co. St. Louis Mo. Vectors pBluescript and pUC18 were obtained from laboratory stocks originally purchased from Stratagene and New England Bio Labs respectively. pSUP401 and pK18 was obtained from Simon *et al.* (1983) and Pridmore (1987) respectively while pJQ200 was created in this laboratory (Quandt and Hynes, 1993). Subclones of the two *fixG* operons were produced to facilitate manipulation as were constructs to evaluate operon activity and functional necessity (see Table 2.1 and appendix 2). Agarose gels were produced at a weight per volume ratio of 0.8% except for total DNA gels which were 0.5% to enhance large band separation (Sambrook *et al.* 1989) and were run in Tris-borate buffer at varying voltages as required. Agarose for use in electrophoresis analysis was obtained from Gibco BRL as were the molecular weight standards HindIII digested lambda and the 1kb ladder.

2.2 DNA manipulations

After isolating and verifying *fixGHIS* location it became necessary to subclone the two operons to facilitate manipulations. DNA was purified via an alkaline lysis procedure as outlined in Sambrook *et al.* (1989) and digested with various enzymes using the associated buffers provided with each restriction endonuclease. Restriction endonucleases and modifying enzymes were purchased from a variety of sources including Pharmacia, Gibco-BRL, Boehringer-Mannheim, and Promega. DNA fragments were purified from gel slices using the Biorad prep-a-gene kit. Ligations were

Table 2.1. Table of strains and plasmids used for experimentation.

Name	Description	Source
Strain		
R. leguminosarum		
VF39SM	Sm^R	Priefer (1989)
TP2	Lac-, Tc^R	Quandt, This lab
VF39GC-	*fixG*:Nm^R	This work
VF39GD-	*fixG*:Sp^R	This work
VF39GCD-	*fixG*:Nm^RSp^R	This work
VF39*G:lac*	*fixG:lacZ-mob*-Sp^R in TP2	This work
E. coli		
S17.1	RP4 integrated plasmid for mobilisation of *mob* bearing plasmids	Simon *et al.* (1983)
DH 10B	plasmid free strain for transformations	
Plasmids		
pBluescript SK/KS	Cloning vector	Stratagene
pSUP401	Cloning vector	Simon *et al.* (1983)
pUC18	Cloning vector	New England Bio Labs
pK18	Cloning vector	Pridmore (1987)
p1918	Cloning vector	Schweizer (1993)
pJQ200SK	Gene replacement vector	Quandt & Hynes (1993)
pUC18LMS	*lacZ-mob-Sp* cassette	This lab
pLZC3	*R. meliloti fixG* fragment	Kahn *et al.* (1989)
pDD27	*R. meliloti fixHI* fragment	Kahn *et al.* (1989)
interposons	Antibiotic cassettes for gene inactivation	Fellay *et al.* (1987)
pFG92	11.4 kb EcoRI insert of C plasmid *fixNOQP* and *fixGHI* region in pBluescript	This work
pFG92-1	1kb BamHI subclone of pFG92 containing *fixP* in pBluescript	This work
pFG92-4	4kb BamHI subclone of pFG92 containing *fixGHI* in pK18	This work
pFG92-4Blu	pFG92-4 BamHI fragment in pBluescript	This work
pFG92-4mp18	4kb BamHI fragment of pFG92-4 inserted into pJQ200mp18	This work
pFG92-4mp18Sp	Sp cassette placed into XhoI site of pFG92-4mp18	This work

Table 2.1 continued

Name	Description	Source
pFG92-4a	1.8kb BamHI/XhoI fragment of pFG92-4 containing *fixG* in pBluescript	This work
pFG92-4alac	*lac-mob-Sp* SmaI cassette inserted into XhoI site of pFG92-4a	This work
pFG92-4b	2.2kb XhoI/EcoRI fragment of pFG 92-4 in pBluescript	This work
pFG93	4.6kb BamHI fragment of D plasmid *fixP* and *fixGHI* region in pUC 18	This work
pFG93Blu	pFG93 BamHI fragment in pBluescript	This work
pFG93BluRev	pFG93 BamHI fragment in reverse orientation	This work
pFG93-1	1kb ClaI/EcoRI fragment of pFG93 containing *fixHI* in pBluescript	This work
pFG93-2	1.4kb EcoRI/HindIII fragment of pFG93 containing *fixP* in pBluescript	This work
pFG93-3	2.0kb HindIII/ClaI fragment of pFG93 containing *fixGH* in pBluescript	This work
pSUP401-93	pFG93 EcoRI fragment in pSUP401	This work
pSUP401-93Sp	Sp cassette inserted into SalI site of pSUP401-93	This work
pFG93BluSp	pSUP401-93Sp EcoRI fragment in pBluescript	This work
pFG93SpJQ	pFG93BluSp XhoI/SstI fragment into pJQ200SK	This work
pFG93-4	1kb EcoRI/BamHI cosmid fragment containing *fixIS* in pBluescript	This work
pFG93-5	1kb BamHI/XhoI cosmid fragment containing *fixS* in pBluescript	This work

carried out in 5 microlitre volumes and transformations were carried out according to Sambrook *et al.* (1989). The C plasmid copy of the *fixG* operon was subcloned via BamHI digests to isolate the operon (pFG92-4, see Table 2.1) and this region was cloned into the gene replacement vector pJQ200. This allowed the digestion and blunt ending of the unique XhoI site for insertion of a Nm antibiotic cassette from the omega fragments (Fellay *et al.* 1987). pFG92-4a was a BamHI/XhoI subclone of pFG92-4 and was used in the creation of the *lacZ* reporter construct. pFG92-4a was digested with XhoI, end filled with the Klenow fragment, and ligated to the SmaI fragment of pUC18LMS, a vector containing the *lacZ-mob-Sp* cassette to be used as a reporter gene. This element included a mobilisation point for transfer into *Rhizobium* cells so further manipulations were unnecessary. Nested deletions of this operon were produced by digesting pFG92-4Blu with KpnI as the protected end and EcoRI to expose a 5' segment for digestion with ExoIII.

The D copy of the *fixG* operon was also subcloned to simplify further manipulations. When it became apparent that gene replacement would not be possible by using pFG92-4mp18 with different antibiotic cassettes a pFG93 based vector was produced. The EcoRI fragment of pFG93 was placed into pSUP401 to utilise the unique SalI site at the end of the *fixG* gene. The Sp antibiotic cassette isolated from the omega fragments was inserted into p1918 (which contains two multiple cloning sites adjacent but inverted to one and other) (Schweizer, 1993) via a HindIII digest. This produced a Sp cassette flanked by SalI sites which could be used. The result, pSUP401-93Sp, was placed into pBluescript via EcoRI and removed from this vector and inserted into the gene replacement vector pJQ200SK with a XhoI/SstI digest and religation. Nested deletions of this operon were conducted on pFG93Blu, pFG93BluRev and pFG93-3. In all cases the construct was digested with SstI to protect the priming site while XbaI was used to create the 5' site for digestion.

Total DNA preparations of *R. leguminosarum* were carried out according to an amended protocol of Meade *et al.* (1982) (see

appendix 1) and digested with EcoRI overnight. The DNA was then run on a 0.5% agarose gel at 20 volts for two days to achieve the desired band separation. This allowed the verification of insertion of the disrupted operon by band size alterations which could be seen via Southern hybridisations.

2.3 Southern Hybridisations

In order to identify the *fixG* operons of *R. leguminosarum* Southern blots were carried out using Zeta Probe membranes purchased from Biorad and probed using the *fixG* probe (pLZC3) and the *fixHI* probe (pDD27) provided by Dr. Kahn *et al.* (1989). Blotting onto membranes was done via an alkaline blotting technique as outlined by the Biorad protocol for the Zeta Probe membranes. Immobilisation of the DNA onto the membrane was achieved via a capillary transfer system after which the Zeta Probe membrane was rinsed in 2xSSC and dried in a vacuum oven at 80°c for thirty minutes. Non-radioactive probes were produced by isolating fragments from gels according to the Biorad prep-a-gene kit instructions and the random hexanucleotide primer method using digoxigenin-labeled deoxyuridine-triphosphate extension as outlined in the Boehringer Mannheim DIG protocols. Southern hybridisations with the *fixG* and *fixHI* probes of *R. meliloti* was carried out at 65°c to maximise binding efficiency with a nonhomologous probe while 68°c was utilised for all hybridisations with *R. leguminosarum fixG* (pFG93-3) probes to eliminate background contamination.

2.4 Nested deletions and sequencing

Nested deletions were produced with a kit purchased from Pharmacia and an amended protocol from that supplied by Pharmacia Biotech. DNA for nested deletions was prepared according to the ABI protocol for sequencing grade DNA and this was then digested with the 3' cutting enzyme and complete digestion was verified on an agarose gel. The DNA was then ethanol precipitated and digested with the 5' cutting enzyme. After this set

of digests, a sample of the DNA was digested with 1 unit of ExoIII at 37°c for 15 minutes and run on a gel along with untreated linearized DNA. This ensured production of DNA receptive to nested deletion reactions. Deletions were carried out at 37°c with 4 microlitres of 0.3M NaCl added to the ExoIII buffer and samples removed at 1 minute intervals and treated as outlined in the Pharmacia Biotech protocol for nested deletions. DNA for sequencing was produced according to the instructions outlined in the ABI sequencing protocol except for the addition of two phenol-chloroform (50:50) extractions rather than two chloroform extractions. The deletions were sequenced using the ABI automated sequencer located at the Regional DNA Synthesis Lab, Foothills Hospital where ABI reagents for PCR sequencing and gel analysis were used. Primers used for sequencing were produced in the Regional DNA Synthesis Lab and included the T7 (5'-AATACGACTCACTATAG-3'), M13 forward (5'-GTAAACGACGACGGCCAGT-3'), and T3 (5'-ATTAACCCTCACTAAAG-3') primers. Sequence results were analysed via the Seq-Ed program provided by Applied Biosystems Inc. and the data was then compared to Genbank and other libraries through the Blastx and Blastn programs (Gish *et al.* 1993). Nested deletions were aligned by the program Macaw produced by Greg Schuler of the National Center for biotechnology information, and the final DNA sequence was placed into the program DNA Strider written by Christian Marck to identify digest points and produce protein sequences.

2.5 *R. leguminosarum* manipulations

After mutations of the *fixG* operon had been produced using the omega interposons obtained from Dr. Fellay (Fellay *et al.* 1987) they were inserted into the gene replacement vector pJQ200 and transformed into *E. coli* strain S17-1 which carries the genes for mobilising *mob* bearing plasmids. A surface mating (O'Connell, 1984) was then carried out on TY medium with recipient *R. leguminosarum* VF39 cells and left overnight. The mating sample was then collected, resuspended in distilled water and plated on

medium containing Sm, the antibiotic matching that of the omega interposon, and sucrose to activate the *sacB* gene into producing levan, a polymer lethal to Gram negative bacteria. All surviving cells now containing the mutated *fixG* operon were replica plated on Gm to eliminate single recombinants and all Gm sensitive clones were kept. Double mutations were created by taking VF39GC- strains and introducing the pRleVF39d mutant construct pFG93SpJQ to obtain gene replacements in the D plasmid. Insertion of the *lacZ* reporter gene developed in this laboratory was done by placing the *pfixG:lacZ-mob-Sp* construct located in the pBluescript vector (pFG92-4alac) into *E. coli* strain S17.1 and carrying out a surface mating with the Lac- *R. leguminosarum* strain TP2. The mating was then collected, rinsed with sterile distilled water and plated on Sm/Sp bearing medium.

2.6 Beta-galactosidase assays

VF39*G:lac* and TP2 strains were inoculated into VMM media containing 0.05% nitrate and 3 different carbon sources (1.0% succinate, 1.0% glucose, or 1.0% mannitol) to evaluate their effect on *fixG* expression. After three days of aerobic growth at 28°c the tubes were left unagitated overnight to create a microaerobic environment after which time assays were carried out. Aerobic and anaerobic expression were compared by carrying out tests with 1.0% succinate in VMM tubes with 0.05% nitrate and either shaken or left still at 28°c for four days after which time assays were carried out. Copper induction was also tested with different copper concentrations in VMM tubes containing 1.0% succinate and 0.05% nitrate after an aerobic growth period of three days followed by an overnight microaerobic incubation at 28°c after which time assays were carried out. Beta-galactosidase assays were conducted according to Sambrook *et al.* (1989) and assayed using a Spectronic 21 from Bausch and Lomb. Beta-galactosidase assays were analysed using the Systat program written by Leland Wilkinson for statistical significance and CricketGraph from Computer associates international inc. for presentation purposes.

2.7 Plant tests

Trapper pea seeds (*Pisum sativum*) were obtained from Chin Ridge Seed Processors Ltd. of Taber Alberta and were sterilised by rinsing and agitating in 70% ethanol for 10 minutes, a 50% bleach rinse for 10 minutes followed by 5 rinses in distilled water and left overnight in sterile distilled water and darkness. They were then placed on TY medium at 28°c to allow germination and verify sterility. After three days these seedlings were then placed in 500ml flasks containing 250ml of sterile vermiculite purchased from Vil vermiculite Inc. Toronto Ontario and 100ml of plant medium (see appendix 1). They were then inoculated with the appropriate strains of *R. leguminosarum* VF39, another 100ml of sterile plant medium and grown for five weeks in an illuminated growth chamber (16 hours light, 8 hours dark) produced by Conviron of Winnipeg Manitoba.

The plants were then collected and divided into root and stem sections for further analysis. The stems were dried down in a vacuum oven for three days and their dry weights were recorded. The root systems were appraised for nodule presence and then placed in stoppered 250 ml bottles containing 5ml of acetylene. After a one hour incubation time at room temperature, a 5 ml sample volume was removed with a needle and syringe and injected into a vacuum tube. A 2 ml sample of this volume was then removed and injected into a Varion 3700 gas chromatographer located in the laboratory of Dr. Reid to measure relative acetylene and ethylene concentrations. Plant dry weights were analysed using Systat for statistical significance and CricketGraph for presentation purposes. To verify that no contamination had occurred, several nodules from each test group along with the VF39 control sample were collected and the contents of the nodules released into sterile distilled water. This solution was then plated on TY plates bearing Nm, Sp, NmSp, and no antibiotics. Bacteria isolated from the single mutant inoculated plants only grew on TY and TY containing the antibiotic corresponding to the antibiotic

cassette of that single mutant. Cells isolated from the double mutant inoculated plants were capable of growing on all the plates they were spread on. Colonies of each single mutant and the double mutant were then isolated and their total DNA recovered in order to perform a Southern hybridisation to verify mutational integrity.

Chapter 3. Results and Discussion

3.1 Identification of *fixGHIS* operons

A *fixG* probe isolated from *R. meliloti* was used against *R. leguminosarum* VF39 total DNA isolated from strains cured of certain plasmids. Two regions showed homology to the probe one residing on the C plasmid while the second was located on the D plasmid indicating that *R. leguminosarum* contains two *fixG* operons (see figure 3.1). In other members of the Rhizobium genera *fixG* was found downstream of the *fixN* operon and four clones of a cosmid library had been isolated which contained elements of the *R. leguminosarum fixN* operon. Based upon conservation of genomic arrangements in the nitrogen fixers these cosmids were screened with the probes pLZC3 and pDD27 isolated from *R. meliloti* (Kahn *et al.* 1989) to detect *fixG* and *fixHI* regions respectively. Four cosmids were identified as containing the *fixG* operon (see figure 3.2) and these cosmids were used as the source of the *fixG* operons for further manipulations. These regions were subcloned, mapped with restriction endonucleases, and probed again with pLZC3 and pDD27 to localise the genes to facilitate further manipulations (see figure 3.3). The pRleVF39c copy of the *fixG* operon was isolated via a BamHI digest to produce pFG92-4 while the second copy located on pRleVF39d was isolated using EcoRI to produce pFG93.

3.2 Operon inactivation

Once clones of the DNA corresponding to *fixG* homologues had been isolated, omega interposons (Fellay *et al.* 1987) were inserted into the two operon copies. The C copy of *fixG* was inactivated by digesting pFG92-4 and the omega fragment containing the neomycin resistance cassette with XhoI and ligating the fragments (see figure 3.4). The D copy operon was disrupted by removing the spectinomycin resistance cassette placed in p1918 with SalI and inserting it into the unique SalI site of pSUP401-93 (see figure 3.4). The gene replacement vector pJQ200 was then utilised to create vectors to exchange the wild type *fixG* operons of *R. leguminosarum*

Figure 3.1. Southern hybridisation of total DNA digested with EcoRI showing the two *fixG* operons found in *R. leguminosarum.* The strains cured of either the C and D plasmids have a single band at 6.0kb and 11.0kb respectively while all other strains maintain both bands. The control lane is the fragment used to produce the probe itself, pLZC3 (Kahn *et al.* 1989).

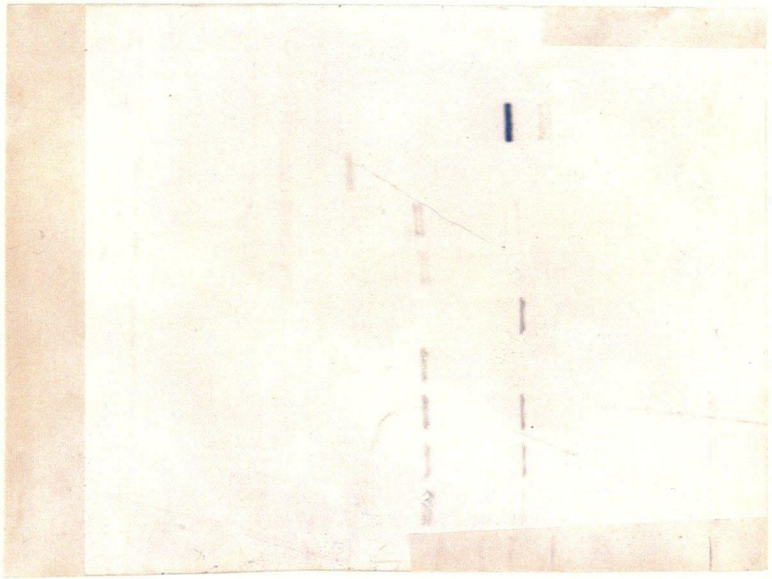
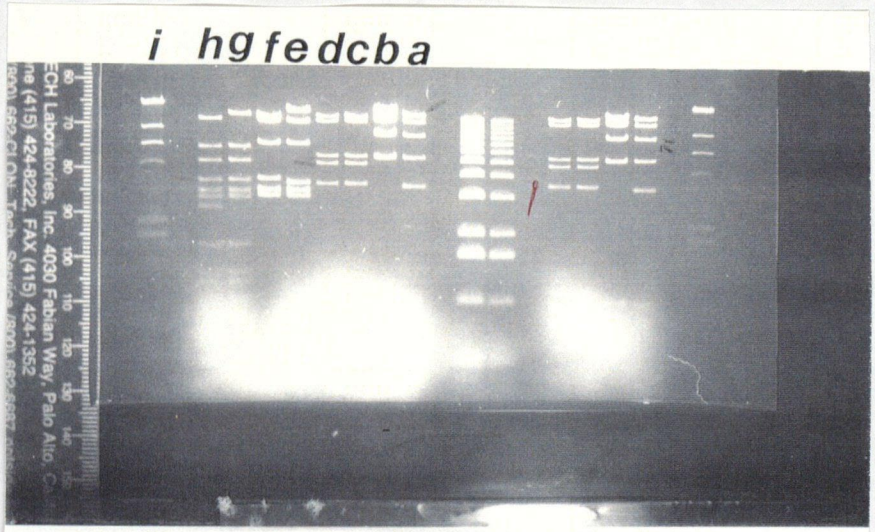

controls
f cured
e cured
d cured
c cured
b cured
a cured
VF39

Figure 3.2. Southern hybridisation using the probe pLZC3 and the cosmid library containing the *fixG* operons of pRleVF39c (LB3 and 4) and pRleVF39d (LB 1 and 2). Both the gel and the blot show LB 4, 3, 2, and 1 digested with BamHI (lanes a, b, c, and d respectively) and digested with EcoRI (lanes e, f, g, and h respectively). Lane i contains the lambda HindIII marker.


i hgfedcba


a b c d e f g h i

Figure 3.3. Restriction map of associated regions of pRleVF39c and pRleVF39d showing the reiterated operons *fixNOQP*, *fixGHIS* and the single *fixLJK* locus. B: BamHI, C: ClaI, E: EcoRI, H: HindIII, N: NotI, P: PstI, S: SalI, S: SmaI, X: XhoI.

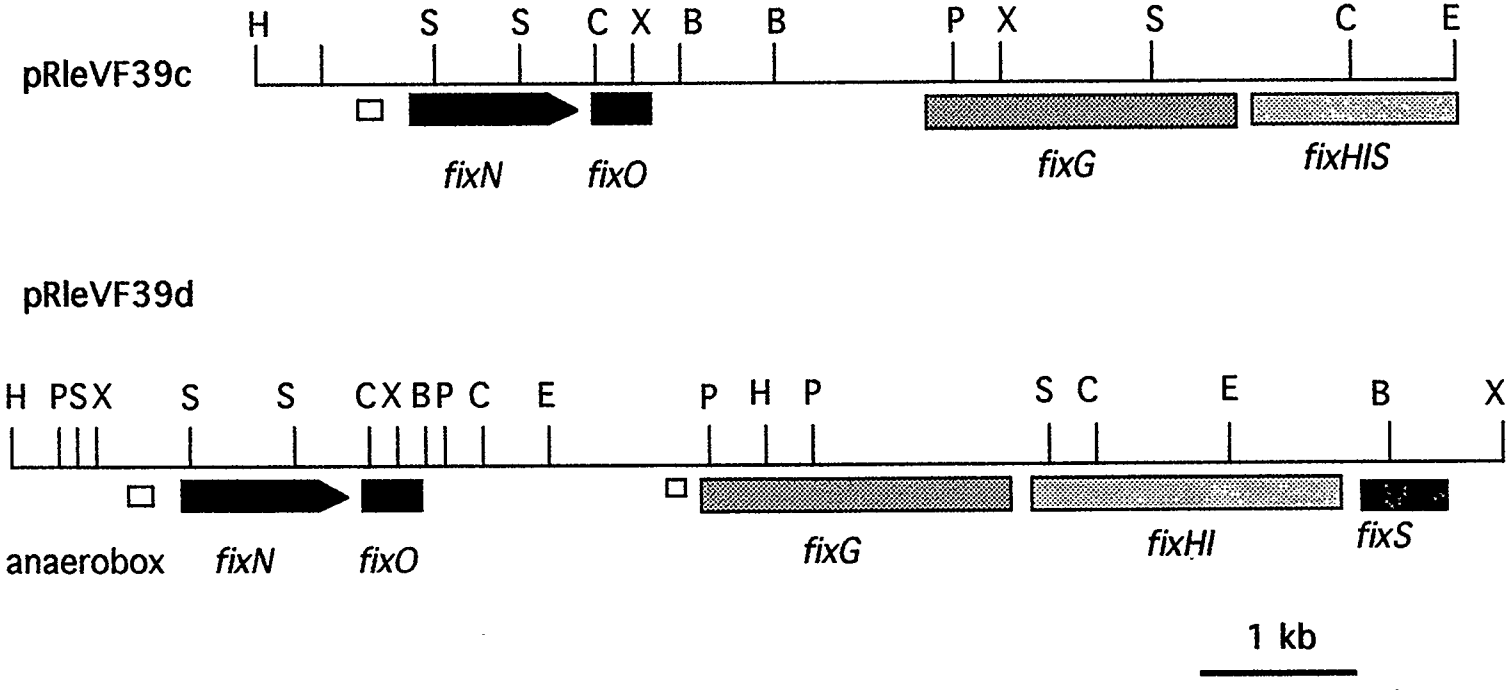
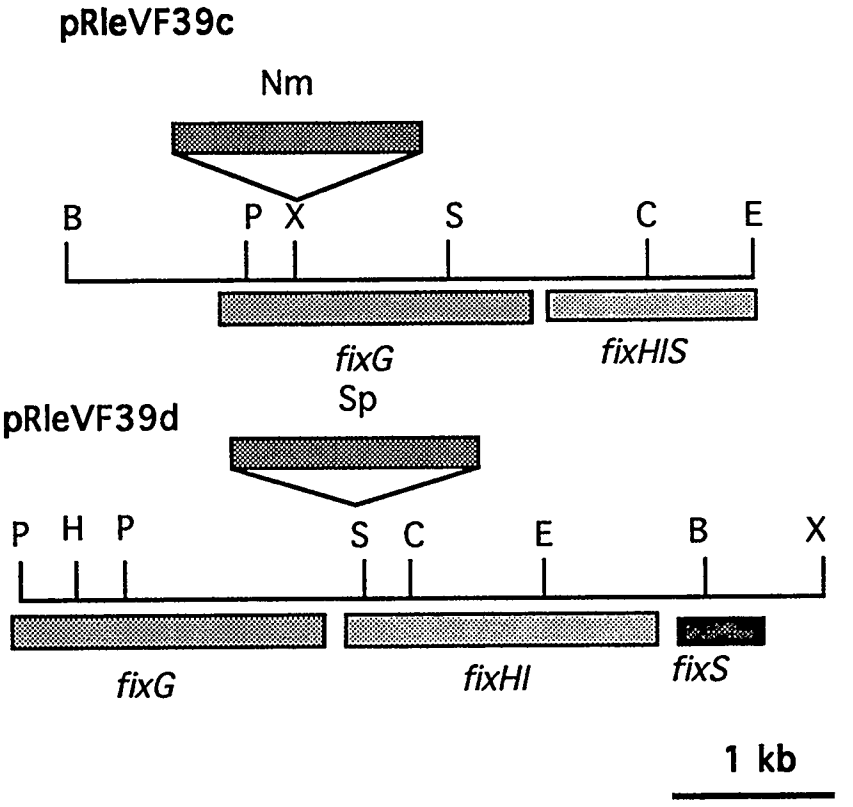

pRleVF39c
H
S
S
C
X
B
B
P
X
S
C
E
fixN
fixO
fixG
fixHIS
pRleVF39d
H PSX
S
S
CXBP C
E
P
H
P
S C
E
B
X
anaerobox
fixN
fixO
fixG
fixHI
fixS
1 kb

Figure 3.4. Inactivation of the *fixG* operons by antibiotic cassette insertion at the unique XhoI site of the C copy and the SalI site of the D copy of strain VF39. B: BamHI, C: ClaI, E: EcoRI, H: HindIII, P: PstI, S: SalI, X: XhoI.


pRleVF39c
Nm
B
P
X
S
C
E
fixG
fixHIS
pRleVF39d
Sp
P
H
P
S
C
E
B
X
fixG
fixHI
fixS
1 kb

VF39 with the mutated operons. pFG92-4 was inserted into pJQ200mp18 with a XhoI/SstI digest to make pFG92-4mp18 while pFG93BluSp was inserted with a XhoI/SstI digest to create pFG93SpJQ (see figure 3.5). These constructs were transformed into *E. coli* strain S17.1 which has genes capable of mobilising *mob* bearing plasmids. These inactivated operons were then introduced into the genome of wild type *R. leguminosarum* cells via a surface mating to produce strains VF39GC- and VF39GD- with the operons of the C and D plasmids disrupted respectively. Once single mutations had been produced a double mutant was created by taking strain VF39GC- and introducing the gene replacement vector pFG93SpJQ to produce strain VF39GCD-. Initial attempts to utilise a single gene replacement vector using pFG92-4mp18 and several antibiotic resistance cassettes failed to generate mutations in the D copy of C mutants or strains cured of the C plasmid. This may have been due to insufficient homology between the two operons to allow recombinational events to occur, the flanking regions surrounding the antibiotic cassette were of an insufficient length to allow crossing over, or a combination of the two elements. In any event a second gene replacement vector was necessary. Once mutant strains had been produced they were verified by isolating total DNA from them, digesting it with EcoRI, and performing a Southern hybridisation with a *fixG* probe. Increases in the size of the *fixG* operons equivalent to the insertion of the antibiotic cassette verified operon inactivation.

3.3 Plant test results

Surface sterilised *Pisum sativum* seeds were inoculated with wild type *R. leguminosarum* strain VF39, single mutants VF39 GC-, VF39 GD-, and the double mutant VF 39 GCD-. After five weeks of growth under nitrogen starved conditions the plants were collected and dry weights recorded (see figure 3.6 and figure 3.7). Several nodules were also collected from each plant and *R. leguminosarum* cells from these nodules were isolated and screened on selective media to verify that no cross contamination had occurred.

Figure 3.5. Insertion of inactivated *fixG* operons into the gene replacement vector pJQ200 for introduction into the wild type strain VF39 to produce single and double mutations.

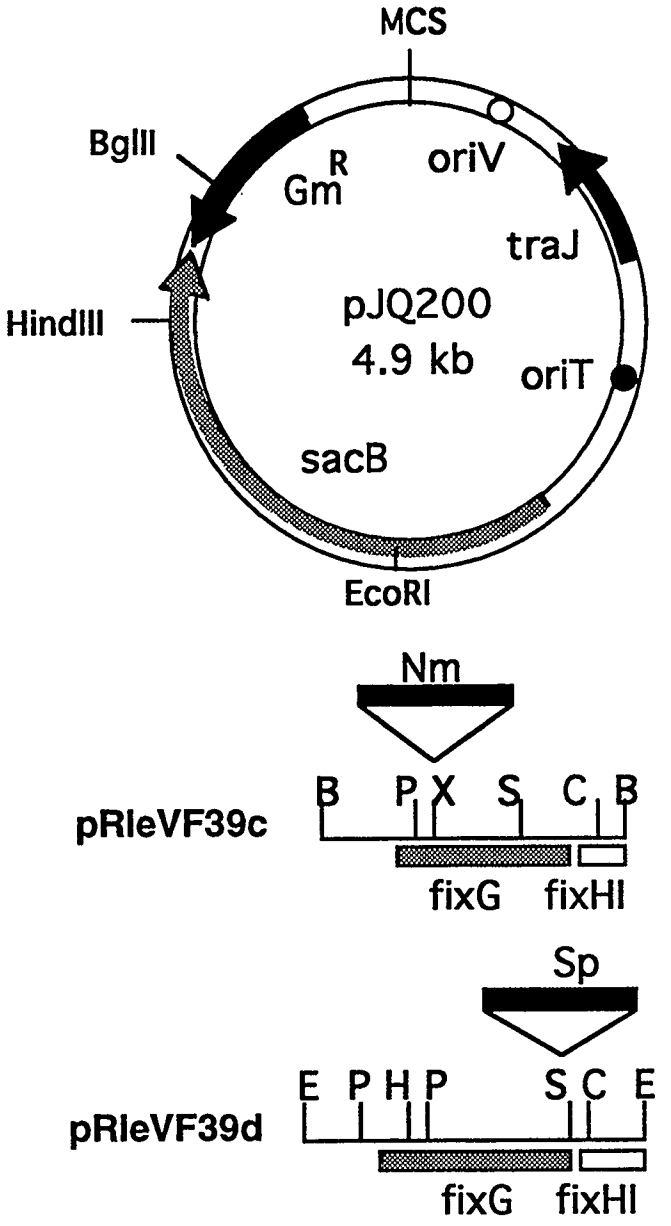
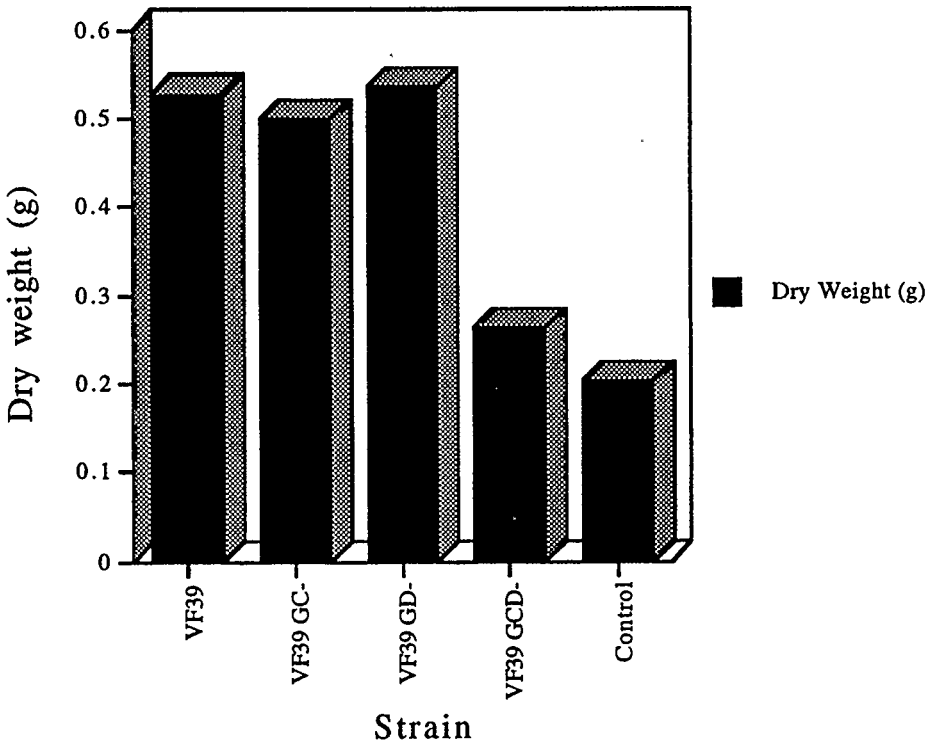

MCS
BglII
HindIII
Gm R
oriV
traJ
pJQ200
4.9 kb
oriT
sacB
EcoRI
Nm
pRIeVF39c
B P X S C B
fixG fixHI
Sp
pRIeVF39d
E P H P S C E
fixG fixHI

Figure 3.6. Photograph showing effects of single and double mutations of the *fixG* operon on pea plants after 5 weeks of growth as compared to wild type VF39 and uninoculated controls.


Control
C-D-
C-
D-
VF39

Figure 3.7. Dry weights of pea plants inoculated with VF39, VF39GC-, VF39GD-, VF39GCD-, and distilled water.


0.6
0.5
0.4
0.3
0.2
0.1
0
Dry weight (g)
VF39
VF39 GC-
VF39 GD-
VF39 GCD-
Control
Strain
Dry Weight (g)

A Southern hybridisation (see figure 3.8) was carried out on total DNA samples isolated from the colonies removed from the plants to verify mutational integrity. The single mutant inoculated plants were very close in size and weight to the wild type inoculated plants while plants treated with VF39GCD- resembled the uninoculated control plants (see figure 3.6). Statistical analysis using the Systat program verified these observations and indicated that the variations in plant weight were significant. The root systems of these plants were isolated and subjected to acetylene reduction assays to determine nitrogenase activity (see table 3.1). The plants inoculated with the wild type strain along with those inoculated with the single mutants of the *fixG* operon maintained nitrogenase activity while the plants inoculated with the double mutants lack the ability to reduce acetylene to ethylene. From these tests we have determined that at least one of the *fixG* operons is required for the formation of a Fix^+ phenotype and that the elimination of one operon does not impede nitrogen fixation or plant growth.

The phenotype of *fixG* operon mutants in different nitrogen fixers is extremely variable. In *R. meliloti* and *B. japonicum* any alteration of this region results in a Fix^- (Kahn *et al,* 1989, Preisig *et al,* 1993) phenotype while in the free living nitrogen fixer *R. sphaeroides* disruption of the *fixG* homolog *rdxA* does not impair nitrogen fixation (Neidle and Kaplan, 1992). Between these two extremes *A. caulinodans,* a bacterium capable of fixing nitrogen both *in planta* and as a free living bacterium showed little reduction in nitrogenase activity after mutation of the *fixG* operon under free living or symbiotic conditions (Mandon *et al,* 1993). With these observations we can make the following conclusions; first, the *fixG* operon is required for nitrogen fixation in the obligate symbionts *R. meliloti, B. japonicum,* and *R. leguminosarum.* The unusual results with *A. caulinodans* may reflect the unusual physiological abilities of this bacterium to fix nitrogen under two different conditions but since disruption of *fixI* did lower nitrogenase function 10 fold under symbiotic conditions it can be concluded that this gene is necessary

Figure 3.8. Southern hybridisation of total DNA showing band migration due to insertion of the antibiotic cassettes into the *fixG* operon. VF39 GC- (lane **A**), VF39 GD- (lane **B**), VF39 GCD- (lane **C**) prior to plant tests, VF39 wild type (lane **D**) and 27-1 lacking the D plasmid (lane **E**) are shown as comparisons. Strains VF39 GC- (lane **F**), VF39 GD- (lane **G**), and VF39 GCD- (lane **H**) recovered from the inoculated plants after five weeks of growth. The single and double mutant inoculated plants show no contamination by wild type or other mutated strains verifying sample integrity.

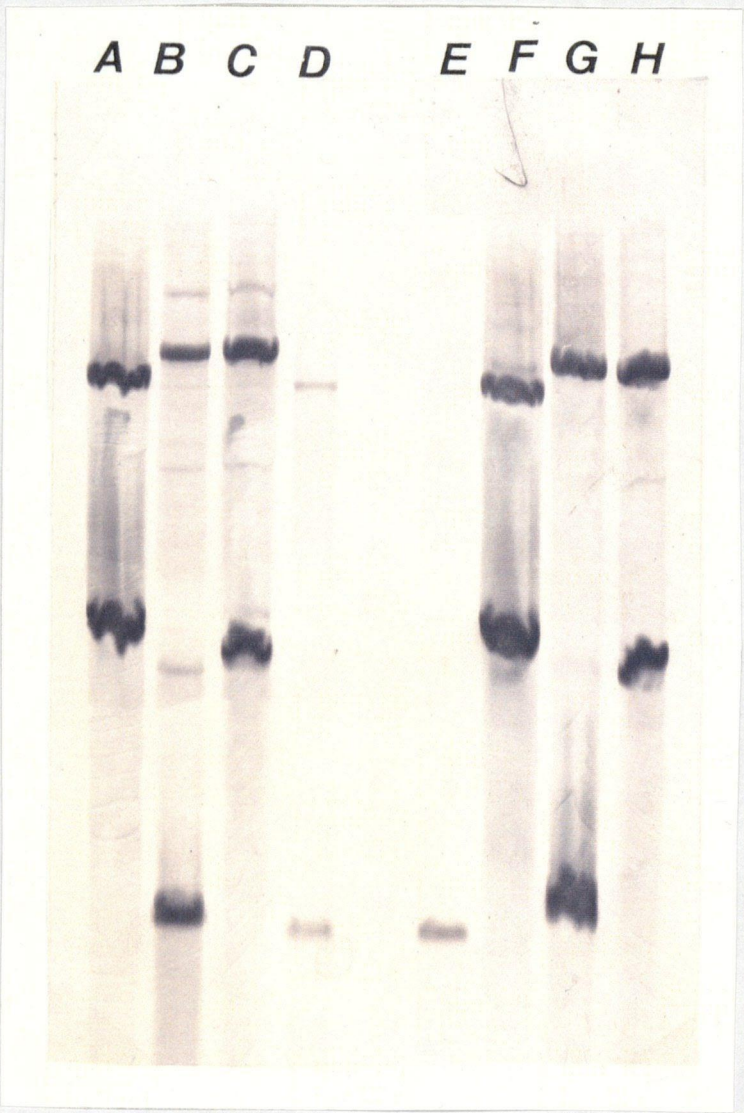

A B C D E F G H

Table 3.1. Acetylene reduction assay results to determine nitrogenase activity of nodules isolated from pea plants inoculated with VF39, VF39GC-, VF39CD-, and VF39GCD-. The + and- symbols represent ethylene production above and below baseline values respectively.

INOCULATION STRAIN	NITROGENASE ACTIVITY
VF39	+
VF39 GC-	+
VF39 GD-	+
VF39 GCD-	-

for maximal nitrogen fixation *in planta.* It may be that the free living nitrogen fixers do not require the cation pump coded for by the *fixG* operon and are able to obtain this ion without a specialised import system. Thus since these genes are conserved among the Rhizobium genus there must be a common physiological requirement for their existence in the symbiotic environment. *R. leguminosarum* contains two of these *fixG* operons but only one is required for successful nitrogenase activity. Disruption of either copy does not effect plant growth or nitrogenase activity indicating that either both copies function at partial activity at once, or one copy is preferentially expressed and the other exists as a reserve system but this is as yet unknown.

3.4 Sequence comparisons of the *R. leguminosarum fixG* operon

Nested deletions of the two operons were produced and sequenced. The resulting fragments were aligned using the programs Macaw and Seq Ap to produce the entire *fixG* operon of the D plasmid and sections of the C plasmid copy. The two operon sequences were compared to each other over the area corresponding to the *fixG* region and a high degree of homology was detected with 80% identity at the nucleic acid level. The structural elements of the FixG protein were conserved with the two complete and one half cysteine clusters detected as well as two large hydrophobic domains (see figure 3.9). The complete D copy of the *fixG* operon was then submitted for comparison to the database of sequenced genes using the Blastn program. High degrees of homology were detected with the *R. meliloti fixG* operon (Kahn *et al.* 1989) and these regions are shown in figure 3.10. Due to the degenerate nature of the DNA sequence and possible divergence during evolutionary development the conserved regions vary from 63 to 86% identity and do not cover the entire operon. The highly conserved areas identified by the blast search correspond to the structural coding regions which produce the hydrophobic amino

Figure 3.9. Protein comparison of the FixG products of pRleVF39c (*RLC*) and pRleVF39d (*RLD*) conserved hydrophobic regions are underlined and conserved cysteine clusters and associated regions are in bold type.

RLC RRFFFFFIE-IWPQEFYYVAGLLVMAGFGLFLVT-A-DR--
RLD GRFFFFFIEKIWPQEFYYVAGLLVFMRGFGLFLVTSAVVRA

RLC WCGYACP-TVWVDLFLVVERAIEGDRNARMKLDAGPLS
RLD WCCYTCPQTVWGDLFLVVERAIEGDRNARMKLDR-PYE

RLC FAKVRKRVVKHSIWLLIGVVTGGAWIFYFADAPSLLVSLFT
RLD LCRAGGGVSPH-----IGVTGGAWIFYFADAPSLLVSLFTI

RLC GRAPAAAYTTVAILTATTYVLGGLMREQV**CTYMCP**GTRIQ
RLD GHAPTSS-TDRPSLLRTTYVLGGLMREQV**CTYMCP**WPRIQ

RLC GAMLDENSLVDTYNDWRGEQRSRHAKRAQVKGLPGGD
RLD GAMLDENSLVVTYNDWRGENSGRHAKKALVNGLSVGD

RLC **CVDCNACVAVC**PMGIDIRDGQQME**CITCALCIDACD**GVMDKLG
RLD **CVDCNACVAVC**PMGIDIRDGQQME**CITCALCIDACD**GVMDKLG

RLC KPRGLIAYATLSEYSSNMSLATDEG
RLD KPRGLIAYATLSEYSSNMSLATDEG

Figure 3.10. Nucleotide alignment of the *fixG* operons of *R. leguminosarum* pRleVF39d(RL) and *R. meliloti* (RM). Numbers indicate the nucleotide position relative to the *FixG* promoter of *R. meliloti* and the nucleotides obtained for the D copy of the *R. leguminosarum fixG* operon.

```
RL 217 CCTCTCTACGCACCTCGGAGGAAGGTCTTTCCCAAGCGTACAGAGGGACGATTCCGTCGG 276
       || || ||||     ||  ||||| |||| || ||||||  | ||||| || |||||| |||
RM 306 CCGCTTTACGAGAAGCGTCGGAAGATCTTCCCGAAGCGGGCCGAGGGTCGTTTCCGCCGG 365


RL 277 ATCAAGTGGATCGTCATGCTGGTTACGCTCGGCATCTACTATCTCGCGCCATGGATTAGC 336
        |||| ||| | || |||||||| ||||||||||||||||||||  |||| |||||  ||
RM 366 TTCAAATGGCTGGTGATGCTGGTGACGCTCGGCATCTACTATCTGACGCCGTGGATCCGC 425


RL 337 TGGGATCGCGGGGC 350
       |||||  ||||||||
RM 426 TGGGACCGCGGGGC 439


RL 858 GACGACCTATGTGCTCGGCGGTCTCATGCGAGAGCAGGTGTGCACCTATATGTGCCCGTG 917
       ||| ||||| ||  |||||||  || |||||||||||||| |||||||||||||||||  ||
RM 881 GACCACCTACGTCTTCGGCGGACTGATGCGAGAGCAGGTCTGCACCTATATGTGCCCATG 940


RL 918 GCCACGCATCCAGGGTGCGATGCTCGACGAAAATTCTCTCGTCGTCACCTACAATGACTG 977
       ||| ||||||||||  || ||||| ||||| || || |||||||| |||||||| |||||
RM 941 GCCGCGCATCCAGGCGGCCATGCTAGACGAGAACTCGCTCGTCGTAACCTACAACGACTG 1000


RL 980  GGGGCGAGAACAGCGGTCGCGTCACGCCAAGAAGGCTTTAGTCAATGGCCTATCGGTCGG 1039
        | || | || | ||| ||||| || || ||||||||    |     |||     ||||||
RM 1001 GCGGGGCGAGCCGCGCTCGCGGCATGCGAAGAAGGCGGCTGCGGCCGGCGAGGTGGTCGG 1060


RL 1040 GGATTGCGTGGATTGCAATGCCTGCGTGGCGGTGTGTCCGATGGGAATCGACATTCG 1096
        ||||||||| || ||||| |||||||| || || ||||| ||||| |||||||| ||
RM 1061 GGATTGCGTCGACTGCAACGCCTGCGTTGCCGTCTGTCCCATGGGCATCGACATCCG 1118


RL 1094 TCGTCGACGGACAGCAGATGGAGTGCATCACATGCGCTCTCTGCATTGACGCCTGCGACG 1153
        ||  |||||| |||||| | || |||||||| ||||| |||||||| ||||||||||||
RM 1114 TCCGCGACGGCCAGCAGCTCGAATGCATCACCTGCGCGCTCTGCATCGACGCCTGCGACG 1173


RL 1154 GTGTCATGGATAAGCTCGGAAAGCCTCGCGGCCTGATCGCCTACGCCACGCTGAGCGAAT 1213
        |||| ||||| |||||||||       ||||| |||||| | ||||| |||||  |||| |
RM 1174 GTGTGATGGACAAGCTCGGCCGCGAGCGCGGGCTGATCTCATACGCGACGCTCAGCGACT 1233


RL 1214 ACTCAAGCAACATGTCGCTTGCCAC 1238
        |  ||  ||| ||| | || || ||
RM 1234 ATGCAGCCAATATGGCCCTCGCGAC 1258


RL 2699 CCGCGACGCGCGACATGTTTCATTGGCTCTCGGGGATGATAGCAGCGCCCCCGCTGATTT 2758
        | || || |||||  | || || ||| | ||||   |||| || |  ||  |||||||||
RM 2747 CGGCCACCCGCGATCTCTTCCACTGGATTTCGGCACTGATCGCCGGACCAGCGCTGATTT 2806
```

```
RL 2759 ATGGGGGCGCTTCTCCTTCAAATTCGGTCTGGAA 2792
        |||  |||   |     |  || ||||  |||||
RM 2807 ATGCTGGCCGCTTCTTCTACAAGTCGGCTTGGAA 2840


RL 3511 CGTGCCCGTGGTCCAGGTCGTCGCCGGGGGGAGCTTTTCC 3550
        ||| || || ||||||||||| || |  ||| |  ||| |
RM 3549 CGTTCCGGTCGTCCAGGTCGTTGCGGCTGGGCGGCTTTTC 3588


RL 4259 CCGACAGAACTTCGCTCTTGCCATTGGTAAAAACGTTCTGGCGGTGCCGATCACCATCAC 4318
        ||| |||||||||||| || || || ||  | |||||  | || |||||||||| | ||
RM 4362 CCGGCAGAACTTCGCGCTGGCGATCGGCTACAACGTCATCGCCGTGCCGATCGCAATTCT 4421


RL 4319 CGGATTGGCGACGCCGCTGATAGCGGCGGTGG 4350
         || |  ||||| |||||  | || || || |
RM 4422 TGGCTACGCGACTCCGCTCGTTGCCGCTGTCG 4453


RL 4466 CCGCATGAACATGTTGATCTATCTCATACCCG 4497
        || ||||||||   | ||||||||||| || |
RM 4565 CCTCATGAACACCCTTATCTATCTCATTCCAG 4596
```

acids for membrane insertion and the peptides used for the formation of the cysteine clusters used as redox centers. These areas are needed for the proper functioning of the protein while the linker regions are not as highly conserved and have adopted a divergent sequence High degrees of similarity were also found with the partially sequenced *fixG* gene of *B. japonicum,* (Presig *et al.* 1993) the *RdxA* gene of *Rhodobacter sphaeroides* (Neidle and Kaplan, 1992), and the *fixGHI* region of *A. caulinodans* (Mandon *et al.* 1993) (see figure 3.11). The searches revealed a highly conserved area found in all *fixG* homologs upstream of the globular transmembrane domain. This area may represent a short leader sequence for the proper insertion of the FixG protein and its accompanying components into the membrane. The *R. leguminosarum* FixGHIS proteins share several structural components with the *R. meliloti* FixGHIS proteins. All the members of the *fixGHIS* operon code for proteins which have long hydrophobic regions which indicates that the proteins, like those of *R. meliloti* are membrane bound (see figure 3.11). FixG of *R. leguminosarum* also contains two and one half cysteine clusters indicative of ferredoxin proteins and may be a redox protein similar to FixG of *R. meliloti.* All of these deductions are fairly safe as Kahn *et al.* (1989) have already made most of these observations. What was unusual in our results was the appearance of copper pump proteins in our Blast comparisons. Kahn *et al.* (1989) determined that FixI was a P-type ATPase that bore striking homology to the K^+ pump of *E. coli.* Based on this evidence, Kahn *et al.* (1989) suggested that FixI was responsible for importing a cation required for symbiotic nitrogen fixation. Our results support these conclusions and may have identified the cation in question.

Two P-type ATPases involved in copper uptake, CopA and CopB were found in *Enterococcus hirae* that contained metal ion binding motifs and their expression was induced by high or low levels of ambient copper (Odermatt *et al.* 1993). In 1994 two groups identified copper pumps in the cyanobacterium *Synechococcus* 7942. The *pacS* gene was found to have a similar

Figure 3.11. Protein comparison of the FixG leader sequence found in *R. leguminosarum* (*RL*) pRleVF39d, *R. meliloti*, (*RM*) *R. sphaeroides* (*RS*), *B. japonicum* (*BJ*), and *A. caulinodans* (*AC*) (**A**). Protein comparison of the globular transmembrane domain (**B**), the hydrophobic regions (**C**) and the cysteine clusters (**D**) of FixG with conserved cysteines highlighted and the hydrophobic regions of FixH (**E**), FixI (**F**), and FixS (**G**) of *R. leguminosarum* pRleVF39d and *R. meliloti*. Numbers indicate amino acid position within the protein.

A.	*RLD*FixG		PLYAPRRKVFPKRTEGRFRRIKWIVML	
	*RM*FixG	27	PLYEKRRKIFPKRAEGRFRRFKWLVML	53
	*BJ*FixG	18	PLYAARKKVYPQSVSGTFRRIKWGLM	43
	*AC*FixG	22	PLYAARRAIYPQSVHGRLRTTKWVLL	47
	*RS*RdxA	4	PLYAPRTPIFPRQISGAFRTAKWWILA	30
B.	*RLD*FixG		KWIVMLVTLGIYYLAPWISWDRG	
	*RM*FixG	48	KWLVMLVTLGIYYLTPWIRWDRG	70
	*RS*RdxA	25	KWWILAVSLGIYLLTPWLRWDRG	46
C.	*RLD*FixG		PTSSTDRPSLLRTTYVLGGLMREQV	
	*RM*FixG	208	PVAYTTIGILTATTYVFGGLMREQV	232
D.	*RLD*FixG		VCTYMCPW	
	*RM*FixG	232	VCTYMCPW	239
	*RLD*FixG		CVDCNACVAVCP	
	*RM*FixG	281	CVDCNACVAVCP	292
	*RLD*FixG		CITCALCIDACD	
	*RM*FixG	306	CITCALCIDACD	317
E.	*RLD*FixH		FGVVIAVNVTM	
	*RLC*FixH		---VMPVNVTM	
	*RM*FixH	552	FGTVISVNLVM	562
F.	*RLD*FixI		MFHWLSGMIAAPPLIYGGASP	
	*RM*FixI	847	LFHWISALIAGPALIYAGRFF	867
	*RLD*FixI		SPVVHLLALVSFLAWGFLGGD	
	*RM*FixI	1066	SPAVHLLALLTFVGWMLVEGD	1086
	*RLD*FixI		WKQAMLVAVAVLIITCPCAL	
	*RM*FixI	1087	VRHAMLVAVAVLIITCPCAL	1106
	*RLD*FixI		QNFALAIGKNVLAVPITITGLAT	
	*RM*FixI	1382	QNFALAIGYNVIAVPIAILGYAT	1404
	*RLD*FixI		PLIAAVMSTSSIIVVTNAL	
	*RM*FixI	1405	PLVAAVMSSSSLVVVFNAL	1423
G.	*RLD*FixS		LIYLIPIALLMGGIGLLAFLW	
	*RM*FixS	1453	LIYLIPVALSLGGLGLVAFLW	1473

metal binding motif and displayed increased activity in the presence of copper or silver (Kanamaru *et al.* 1994). The similar *ctaA* gene was also isolated and its inactivation resulted in cells with increased copper tolerance indicating that this gene product was responsible for copper import into the cell (Phung *et al.* 1994). All of these gene sequences have the characteristic features and structural elements of metal dependent P-type ATPases. The two *R. leguminosarum* FixI protein sequences were compared to that of the copper pumps and substantial homology was detected (see figure 3.12). FixI of *R. leguminosarum* has a metal binding domain similar in sequence to other copper pumps with a conserved cysteine residue and this N-terminal subunit may be the mechanism for metal detection. The FixI protein also encodes an ion transduction region which is thought to span the membrane and allow import of the cation. Central to this is the presence of two cysteine residues which are conserved in all the copper pumps and this may impart metal co-ordination on the system. Between these cysteine residues lies a highly conserved proline residue. This residue is found in all P-type ATPases and is believed to participate in the conformational change responsible for opening and closing the ion channel. The phosphatase element contains a threonine-glycine-glutamic acid tripeptide which is common to all P-type ATPases and is believed to remove the phosphate from the phosphorylated aspartate residue during the ATPase/transport reaction cycle. The final domain found to have substantial homology with FixI is the hinge region and ATP binding site. Thus from this structural analysis we have suggest that FixI codes for a cation pump as previously reported and the cation being imported may be copper. Copper is a necessary component of cytochromes such as cytochrome a and a3 and would be required in the bacteroid during synthesis of the many electron pathways and respiration systems needed for symbiotic nitrogen fixation.

The promoter element of the *fixG* operon was also sequenced and compared to other promoter elements in different Rhizobium species (see figure 3.13). The sequence comparison shows the

Figure 3.12. Protein alignments comparing the metal binding (**A**), ion transduction (**B**), phosphatase (**C**), hinge and ATP binding domains (**D**) of PacS, CtaA, CopA, CopB, *R. meliloti* (*RM*) FixI, and *R. leguminosarum* (*RL*) pRleVF39d FixI. Conserved regions are underlined and conserved cysteines are highlighted. Numbers indicate amino acid position within the protein.

A.	PacS	9	LRGMG**C**AA**C**AGRIEALIQALPGV	31
	CtaA	20	VEGMK**C**AG**C**VAAVERRLQQTAGV	42
	CopA	12	ITGMT**C**AN**C**SARIEKELNEQPGV	34
	*RM*FixI	43	VPNAY**C**GT**C**IATIEGALRAKPEV	65
	*RLD*FixI		VPTSTAAA**C**ISTIERAFVDASLR	

B.	PacS	383	VGVMIIA<u>CPCAL</u>GL	396
	CtaA	425	ISVLVVA<u>CPCAL</u>GL	438
	CopA	374	VSVLVIA<u>CPCAL</u>GL	387
	CopB	389	VTVFIIA<u>CPHAL</u>GL	402
	*RLD*FixI		VAVLIIT<u>CPCAL</u>VL	

C.	PacS	278	RSTVDESMV<u>TGE</u>SLPVQKQVGD	299
	CtaA	288	QSTLDTAML<u>TGE</u>PLPQPCQVGD	309
	CopA	269	TSALDESML<u>TGE</u>SVPVVEKKEK	413
	CopB	285	HTIVDESAV<u>TGE</u>SKGVKKQVGD	306
	*RLD*FixI		KETWNLSIV<u>TGE</u>SSPVAVASD	
	*RLC*FixI		QSTLDTAML<u>TGE</u>SSP	

D.	PacS	622	LQSRGQVVA<u>MVGDGINDAP</u>ALAQADVGIA	650
	CtaA	657	LQSQGDAVA<u>MIGDGINDAP</u>ALATAAVGIS	685
	CopA	609	LQKAGKKVG<u>MVGDGINDAP</u>ALRLADVGIA	637
	CopB	626	YLDQGKKVI<u>MVGDGINDAP</u>SLARATIGMA	654
	*RLD*FixI		LNGEGRRVL<u>MVGDGINDAP</u>ALATAHVSMA	

Figure 3.13. Comparison of the anaerobox promoter elements and their distance to the ATG start site of different *fix* genes of *R. leguminosarum* (pRleVF39), *R. meliloti, B. japonicum,* and *A. caulinodans.*

pRleVF39c *fixN*	TTGATGTAGATCAA---77bp
pRleVF39d *fixN*	TTGACGCAGATCAA---77bp
R. meliloti fixN	TTGACTTGTATCAA---47bp
B. japonicum fixN	TTGATCTGGATCAA--106bp
A. caulinodans fixN	TTGATTTCAATCAA---70bp
R. meliloti fixK	TTAGTGATCTAA-----61bp
R. meliloti fixG	TTGACGCAGATCAA---65bp
B. japonicum fixG	TTGAGCTGGATCAA---71bp
pRleVF39d *fixG*	TTGATCTGCATCAA---61bp

existence of a classical anaerobox upstream of the transcriptional activation site indicating regulation by oxygen dependent regulatory elements much like the case in other *fix* genes (Batut *et al.* 1989, Colonna-Romano *et al.* 1990, Hynes *et al.* 1992, Preisig *et al.* 1993, Mandon *et al.* 1994). Mutational analysis of the similar *E. coli* Fnr binding site has shown that the first T and the G in the third position are essential for protein binding (Bell *et al.* 1990) and these two bases are conserved among the *fix* genes including *fixG* of *R. leguminosarum.* Since FixK is known to bind to this conserved promoter element and control gene expression in response to oxygen concentrations it is our hypothesis that the *fixG* operon is under the control of either FnrN or the FixK homolog of *R. leguminosarum* and is therefore expressed under microaerobic conditions.

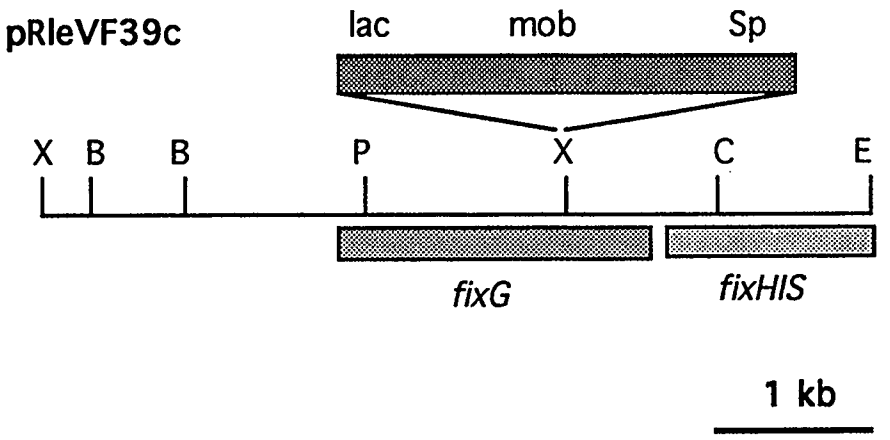
3.5 Activational studies

In order to assess the conditions under which the *fixG* operon is expressed a promoterless *lacZ* gene was inserted downstream of the *fixG* promoter. pFG92-4a was digested with XhoI, blunt ended with the Klenow fragment and a *lacZ-mob-Sp* cassette removed from pUC18 with SmaI was inserted (see figure 3.14). The resulting construct, pFG92-4alac was transformed into S17.1 *E. coli* cells and a surface mating carried out to introduce the plasmid into strain TP2, a *R. leguminosarum* strain with no beta-galactosidase activity. Since the plasmid carrying pFG92-4alac is incapable of replication in a *R. leguminosarum* host the construct was integrated into the genome with a single recombinational event. This strain, VF39 *G:lac* was utilised in a series of experiments to determine operon activation with different carbon sources and different oxygen conditions that would simulate the symbiotic environment.

3.5.1 Succinate induction

Biochemical and genetic studies have indicated that C4-dicarboxylates are probably the carbon and energy source for nitrogen fixation under symbiotic conditions. In order to determine

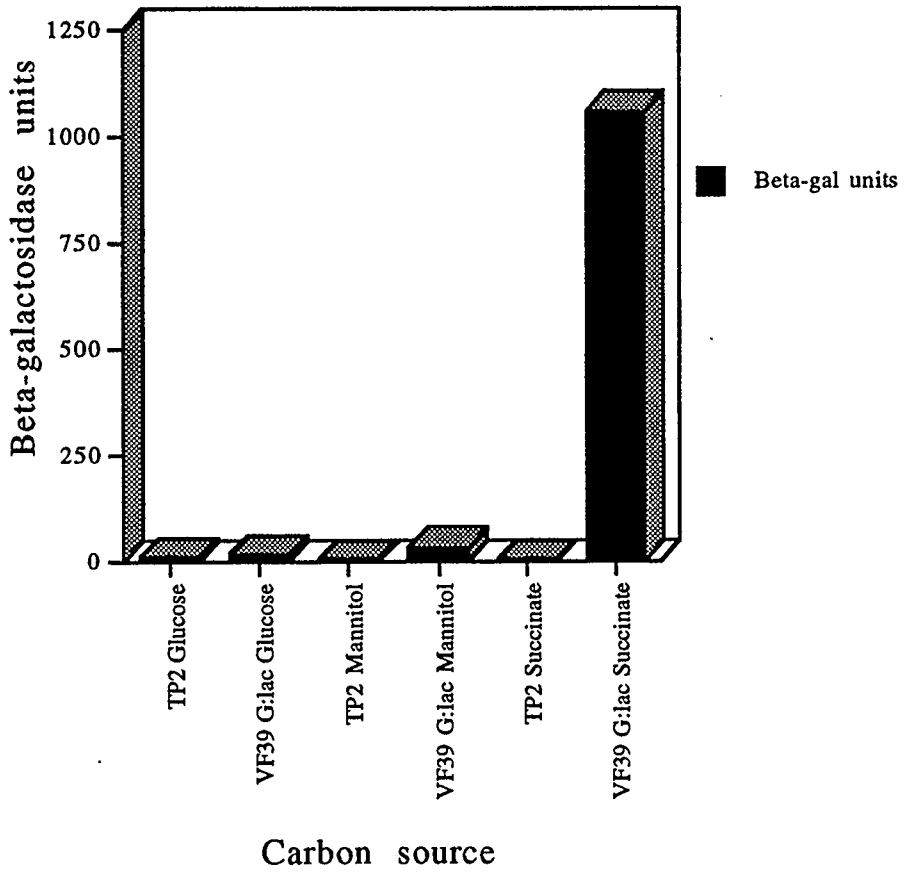
Figure 3.14. Production of strain VF39 *G:lac* by introducing the reporter construct pFG92-4alac into *R. leguminosarum* strain VF39 pRleVF39c plasmid.


pRleVF39c
lac
mob
Sp
X
B
B
P
X
C
E
fixG
fixHIS
1 kb

if the *fixG* operon was affected by carbon sources VF39 *G:lac* and TP2 strains were inoculated into VMM tubes containing 0.05% nitrate and 1% of either glucose, mannitol, or succinate as a nutrient source. After 3 days of incubation at 28°C with agitation to facilitate growth the samples were left to sit overnight at 28°C to induce the formation of a microaerobic environment. Beta-galactosidase assays were then carried out on both the test and control strains (see figure 3.15). The TP2 strain showed no activity with any of the carbon sources while strain VF39 *G:lac* showed no activity with glucose, partial activity with mannitol and greatly increased activity with succinate as the carbon source. This information suggests that succinate activates gene expression in *R. leguminosarum.* It is possible that the heightened gene expression was simply due to enhanced nutrient availability but the cell growth in both glucose and mannitol was greater than that seen in succinate as recorded by A600 readings (data not shown). Therefore the presence of succinate appears to activate the *fixG* operon and possibly other *fix* genes.

Similar observations have also been made in previous studies with other genes and conditions. Microscopic studies have shown that the addition of succinate to free living *Rhizobium* cells causes morphological changes to occur and the cells begin to resemble bacteroids (Urban and Dazzo, 1982, Gardiol *et al.* 1987). These observations and the hypothesis that carbon sources for bacterial energy production are the limiting factor in terms of symbiotic nitrogen fixation has stimulated research into carbon source importation and utilisation. The regulatory and import pathway for C4-dicarboxylates such as succinate, fumarate, malate, and aspartate has been well characterised (Ronson *et al.* 1984). The system is made up of three proteins, a membrane bound sensor DctB, a DNA binding protein DctD, and a second membrane spanning protein DctA. Extra cellular dicarboxylates cause DctB to activate DctD which binds to the UAS of the *dctA* promoter. DctD acts in consort with NtrA and RNA polymerase to initiate transcription and DctA is produced and inserts into the membrane where it acts as a

Figure 3.15. Carbon source activation of the *fixG* operon using the reporter strain VF39 *G:lac* and the carbon sources glucose, mannitol, and succinate.


Beta-galactosidase units
1250
1000
750
500
250
0
TP2 Glucose
VF39 G:lac Glucose
TP2 Mannitol
VF39 G:lac Mannitol
TP2 Succinate
VF39 G:lac Succinate
Carbon source
Beta-gal units

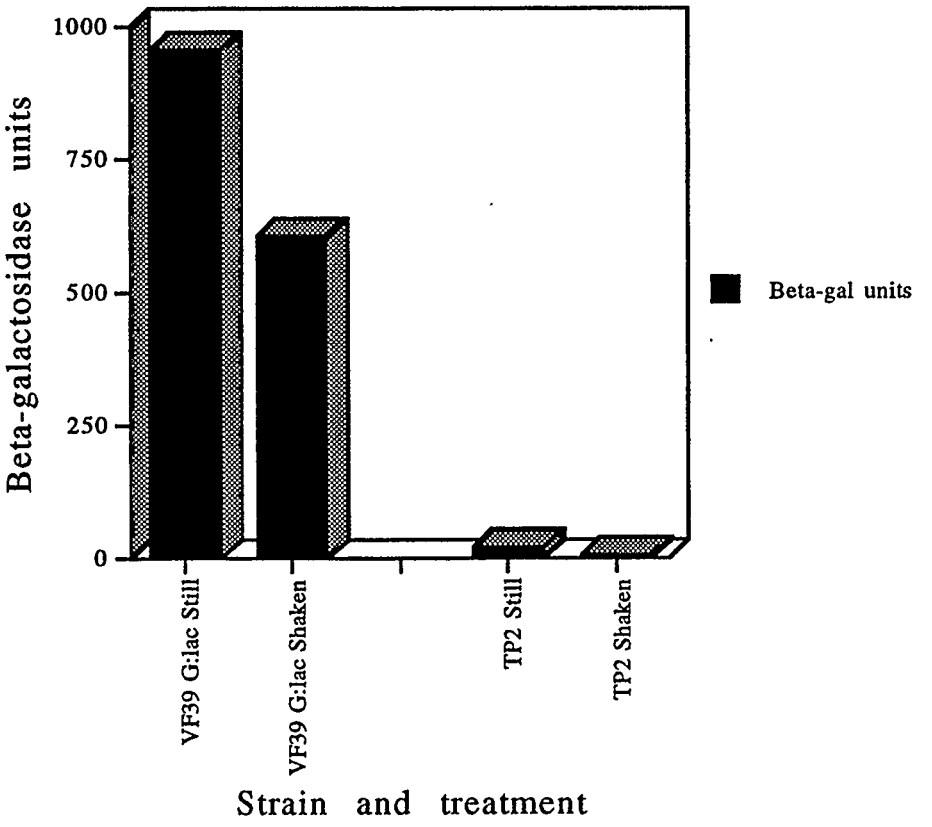
gateway for C4-dicarboxylate transport into the cell. Research into carbon availability has shown that glucose does not stimulate nitrogen fixation by bacteroids but succinate, fumarate and malate does and mutations of the *dct* system allow formation of nodules but no nitrogen fixation (Ronson *et al.* 1987, Finan *et al.* 1983). Mutations for carbohydrate utilisation, in contrast are able to both nodulate their plant hosts and produce a Fix+ phenotype (Watson *et al.* 1988). Based on these observations it has been concluded that dicarboxylate importation into the bacteroid is required for nitrogenase functioning rather than nodule formation and that succinate, fumarate, and malate are required as either a high energy carbon source for nitrogen fixation or for heme biosynthesis. Since heme production has been seen in *dct* mutant strains of *R. meliloti* (Watson *et al.* 1988) this leaves only the first option and attempts to enhance nitrogen fixation *in planta* with overexpressed *dct* mutants have been conducted with minor success. Birkenhead *et al.* (1988) used a 40kb segment of DNA encoding *dct* genes to increase succinate uptake in *B. japonicum* and found increased nitrogenase activity but this may be due to either increased succinate importation itself or an increase in the size of the ATP reductant pool. It is also possible that other unknown regulatory or structural elements were present on the DNA insert as the *dct* region only represents one-seventh of this fragment. Rastogi *et al.* (1992) refined these experiments by only overexpressing the import gene *dctA*. They found no increase in DctA production, succinate importation, or plant dry weights but did see a rise in nitrogen fixation. This may indicate that succinate importation is not the rate-limiting step for nitrogen fixation but is required for a Fix+ phenotype. In terms of specific gene expression *nodD*, the gene regulating *nod* expression for nodulation of the plant host, has been found to undergo a repression in *dct* mutants which allow increased succinate import (Mavridou *et al.* 1995). This may be due to metabolic status of the cell or direct interaction with the Dct regulatory elements or could represent a carbon source induced regulatory pathway.

These facts combined with our observations lead to the following conclusions and ideas for events in the nodule. The C4-dicarboxylates such as succinate are present in the root system and are imported into *Rhizobium* cells via the Dct system and initiate structural changes causing the formation of bacteroids. These carbon sources, either directly or indirectly initiate the reduced expression of the genes necessary for the initial plant-microbe interaction (*nod* genes) and appear to stimulate the genes required for nitrogen fixation *fix* genes). These carbon sources, in addition to their apparent activation role are also required as the energy source for nitrogen fixation. Physiologically these events would seem logical as the genes used for nodulation are no longer required in the nitrogen fixing bacteroid and their reduced expression would save resources. The elevated levels of succinate in the root system would represent a reasonable environmental cue for their inactivation as well as the activator for the nitrogen fixation genes.

3.5.2 Microaerobic induction

The *fixG:lacZ* fusion was also tested under microaerobic conditions with 1% succinate as a carbon source and 0.05% nitrate. VMM tubes were incubated at 28°C for four days under either still or shaken conditions. The VF39 *G:lac* strain showed increased beta-galactosidase activity under microaerobic conditions while the TP2 strain showed no activity in the presence or absence of agitation (see figure 3.16). Several studies have been conducted into oxygen sensing and bacteroid response. In *R. meliloti* the two step oxygen sensing proteins FixLJ regulate the expression of a third gene *fixK* (Waelkens *et al.* 1992) and this gene product is responsible for the expression of several nitrogen fixation genes including *fixNOQP* and *fixGHIS* (Batut *et al.* 1989). Similar regulatory elements were also found in *A. caulinodans* and *B. japonicum* and in each case a *fixK* homolog was found to be under the control of the *fixLJ* gene products (Kaminski *et al.* 1991, Anthamatten *et al.* 1992). In *R. leguminosarum* a gene similar to *fixK* was detected but its predicted protein has additional N-terminal elements similar to the Fnr

Figure 3.16. Effects of aerobic and microaerobic conditions on *fixG* expression using the reporter strain VF39 *G:lac*.


Beta-galactosidase units
1000
750
500
250
0
VF39 G:lac Still
VF39 G:lac Shaken
TP2 Still
TP2 Shaken
Strain and treatment
Beta-gal units

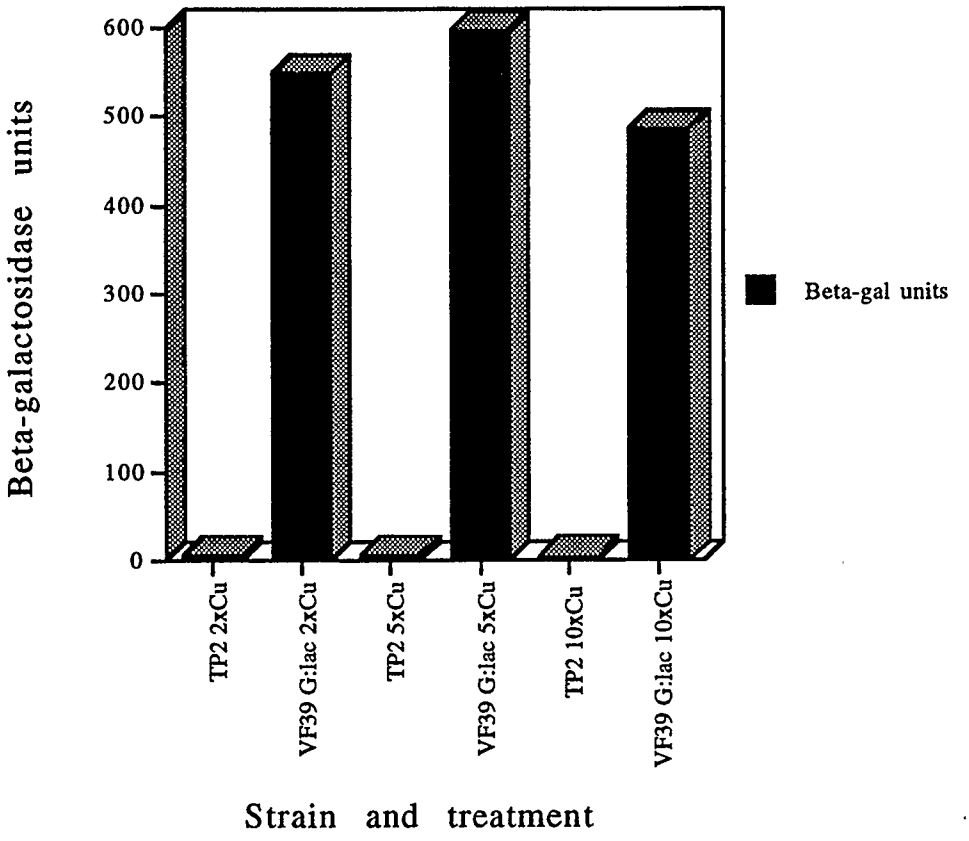
protein of *E. coli* and this may represent a structure for oxygen regulated activity (Colonna-Romano *et al.* 1990). It was also found that this gene, *fnrN* is expressed under low oxygen conditions and the protein itself only mediates transcription under microaerobic conditions (Schlüter *et al.* 1992). A second *fixK* homolog was also found on the C plasmid but its function and importance is unknown (Hynes *et al.* 1992).

It is now known that the microaerobic conditions within the nodule, specifically in interzone II-III, is the environmental cue for the activation of the genes involved in nitrogen fixation (Soupene *et al.* 1995). The *fixK* homologs of the different Rhizobium species are controlled via the oxygen sensor proteins FixLJ and once FixK is produced it acts as a transcriptional activator of several genes. The *hemA* gene of *B. japonicum* has been found to be induced under microaerobic conditions and the effect is mediated via the FixLJ oxygen sensing system (Page and Guerinot, 1995). Among the *fix* genes, the *fixNOQP* operon has been found to be expressed only under microaerobic conditions with FixK required as a transcriptional activator in *R. meliloti* (David *et al.* 1987), *B. japonicum* (Preisig *et al.* 1993), and *A. caulinodans* (Mandon *et al.* 1994). It would appear from our results that the *fixG* operon of *R. leguminosarum* is activated under low oxygen conditions much like the *fixN* operons of other rhizobium species and the *hemA* gene of *B. japonicum.* The genes activated in this pathway all share the same promoter elements (see figure 3.13) such as the anaerobox and this supports the conclusion that they share a similar regulatory element and are therefore part of the same regulatory cascade responding to low oxygen concentrations. Physiologically this hypothesis is reasonable as FixGHIS would be required only for the import of ions during symbiotic nitrogen fixation after passing through the microaerobic interzone II-III and entering the nitrogen fixing zone III. The metal ions imported would then be donated to the cytochromes required for microaerobic respiration coded for by the *fixNOQP* operon which is able to utilise the oxygen donated to it by the leghemoglobin produced by the *hem* genes including *hemA*.

3.5.3 Copper activational studies

Sequence comparisons of the *fixI* gene showed a great deal of homology with previously sequenced copper pumps from a variety of sources. The *copA* gene of *Enterococcus hirvae* was expressed at either high or low levels of ambient copper and appeared to be responsible for its importation (Odermatt *et al,* 1993). Studies with *Synechococcus* species have also found copper import genes such as *ctaA* (Phung *et al,* 1994) and *pacS* whose protein product is located in the thylakoid membrane (Kanamaru *et al,* 1994) and may be responsible for copper import for use in the photosynthetic cytochrome systems. Kahn *et al.* (1989) had already theorised, based on sequence data of the *R. meliloti fixI* gene, that this protein was a membrane bound cation pump. These observations, coupled with the fact that electron transport chains and cytochrome c oxidases such as FixABCX and FixNOQP require copper in their active centers led us to conclude that copper was a good candidate to be the cation imported by FixI. To test this hypothesis, VMM tubes containing 1% succinate, 0.05% nitrate, and different copper concentrations (1x Copper=$5x10^{-8}$g/ml) were used. These tubes were then inoculated with either strain TP2 or VF39 *G:lac* and incubated at 28°C for three days with agitation followed by an overnight incubation without agitation to induce microaerobic conditions. A beta-galactosidase assay was then carried out to determine operon activity at the different copper concentrations. The results (see figure 3.17) indicate that the copper levels used had no effect on *fixG* gene expression. This result was also seen in *R. tropici* studies with the *coxA* gene which is responsible for formation of subunit I of the cytochrome aa3 (Gabel *et al,* 1994). Changes in copper concentration had no effects on gene expression but did appear to have post transcriptional effects detected by spectral alterations in cytochrome aa3. It is possible that a similar system exists with the *fixG* operon in that the genes are expressed irrespective of copper concentrations and the cells simply make do with whatever copper they can acquire.

Figure 3.17. Effects of different copper concentrations on *fixG* activity as measured by the reporter strain VF39 *G:lac.* 2xCu=1x10^{-7}g/ml, 5xCu=2.5x10^{-7}g/ml, 10xCu=5x10^{-7}g/ml.


Beta-galactosidase units
600
500
400
300
200
100
0
TP2 2xCu
VF39 G:lac 2xCu
TP2 5xCu
VF39 G:lac 5xCu
TP2 10xCu
VF39 G:lac 10xCu
Beta-gal units
Strain and treatment

Chapter 4. Conclusions

This work attempted to identify the *fixG* operon in *R. leguminosarum* and determine its function and regulatory elements. Through the course of this study it has been found that two *fixG* operons exist in *R. leguminosarum* strain VF39, one located on the C plasmid while the second is on the D plasmid. Elimination of one of these operons has little effect on nitrogenase activity and plant growth but inactivating both sets of genes results in the loss of nitrogenase activity and reduced plant growth. Sequence analysis has found a great deal of similarity between the two operons within *R. leguminosarum* and also with other nitrogen fixers indicating a necessity for conservation of the genes and their structural elements. Promoter elements of the *fixG* operon show the conserved motifs characteristic of an anaerobox indicating oxygen mediated control of the operon. Other genes bearing this telltale promoter structure are activated via FixK and low oxygen conditions. Our results with a beta-galactosidase reporter system support these previous observations and indicate that *fixG* expression is activated under microaerobic conditions much like those encountered in the nodule. This same reporter system was used in conjunction with different carbon sources and it was found that *fixG* expression was enhanced with succinate. Not surprisingly succinate is one of the major sources of carbon for the bacteroids *in planta* and may represent a nutrient source which delivers the maximum energy for nitrogen fixation. Predictions of the protein structures produced by these operons has suggested that they produce four membrane bound proteins, one containing cysteine clusters indicative of a ferredoxin like protein while a cation pump of the P-type is produced by another gene of the operon. This pump shares a great deal of homology with other prokaryotic pumps used for the import of copper and may serve the same function. Experiments with the reporter gene and different copper concentrations were carried out in order to determine if copper acts as an activator of *fixG* expression. These studies showed no changes

in operon activity and indicate that either copper does not affect *fixG* expression or the copper amounts used in our tests were either too high or too low. With the information garnered from our experiments we may now produce a hypothetical picture of *fixG* activity and necessity. Under microaerobic conditions with succinate as a carbon source, the conditions which exist in the nodule, *fixG* activation occurs. A membrane bound complex made up of four proteins is produced and begins functioning. FixG, a putative redox protein, is coupled to a putative cation pump FixI which is required for symbiotic nitrogen fixation. The redox process is coupled to the pump and allows the importation of copper ions from the plant host. These copper ions are used in the active centers of several cytochromes required for nitrogen fixation to occur. These cytochromes, coded for by *fixABCX* and *fixNOQP*, require copper and iron in order to function and carry out their symbiotically essential role in delivering electrons to the nitrogenase enzyme and allowing respiration in the microaerobic host environment to continue. This theory of the role of the *fixG* operon would explain its necessity for a Fix^+ phenotype *in planta* and the fact that disruption in this operon appears to be overcome by free living nitrogen fixers able to import cations through other means. These studies have revealed both the function of the *fixG* operon and also identified a region of the genome which is expressed only under symbiotic conditions and whose replacement with other genes would not affect nitrogen fixation allowing it to be used as a region for further testing and manipulation.

In terms of future directions this work may take, the expressional control of the operon should be investigated. Sequence data has indicated that the promoter region includes an anaerobox, the FixK binding site it would be of some interest to see if *fixL* or *fixJ* mutants have any effect on *fixG* expression. The relative expression of both *fixG* operons could be measured with two different reporter genes in order to determine if one or both operons are functional at once or if one is preferentially expressed. Cation studies should be continued to determine if another metal

ion is capable of inducing the *fixG* operon and protein purification of the FixGHIS subunits may also be necessary in order to fully characterise the proteins and their functions.

Chapter 5. References

Agron, P., G.S. Ditta, and D.R. Helinski. 1992. Mutational analysis of the *Rhizobium meliloti nifA* promoter. J. Bacteriol. 174: 4120-4129.

Agron, P., G.S. Ditta, and D.R. Helinski. 1993. Oxygen regulation of *nifA* transcription *in vitro*. Proc. Natl. Acad. Sci. USA. 90: 3506-3510.

Anthamatten, D. and H. Hennecke. 1991. The regulatory status of the *fixl*-and *fixJ*-like genes in *Bradyrhizobium japonicum* may be different from that in *Rhizobium meliloti*. Mol. Gen. Genet. 225: 38-48.

Anthamatten, D., B. Scherb, H. Hennecke. 1992. Characterization of a*fixLJ*-regulated *Bradyrhizobium japonicum* gene sharing similarity with the *Escherichia coli fnr* and *Rhizobium meliloti fixK* genes. J. Bacteriol. 174: 2111-2120.

Arigoni, F., P.A. Kaminski, H. Hennecke, and C. Elmerich. 1991. Nucleotide sequence of the *fix*ABC region of *Azorhizobium caulinodans* ORS571: similarty of the *fixB* product with the eukatyotic flavoproteins, characterization of *fixX*, and identification of *nifW*. Mol. Gen. Genet. 225: 514-520.

Batut, J., M-L. Daveran-Mingot, M. David, J.Jacobs, A-M. Garnerone, and D. Kahn. 1989. *fixK*, a gene homologous with *fnr* and *crp* from *Escheria coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. EMBO J. 8: 1279-1286.

Batut, J., E. Santero, and S. Kustu. 1991. In vitro activity of the nitrogen fixation regulatory protein fixJ from *Rhizobium meliloti*. J. Bacteriol. 173: 5914-5917.

Bell, A.I., J.A. Cole, and J.W. Busby. 1990. Molecular genetic analysis of an FNR-dependent anaerobically inducible *Escherichia coli* promoter. Mol. Microbiol. 4: 1753-1763.

Beynon, J.L., M.K. Williams, and F.C. Cannon. 1988. Expression and functional analysis of the *Rhizobium meliloti nifA* gene. EMBO J. 7: 7-14.

Bezdicek, D.F. and A.C. Kennedy. 1988. Symbiotic nitrogen fixation and nitrogen cycling in terrestrial environments. p241-261. in Microorganisms in action, J.M. Lynch and J.E. Hobbie (ed.) Blackwell Scientific, Boston.

Birkenhead, K., S.S. Manian, and F. O'Gara. 1988. Dicarboxylic acid transport in *Bradyrhizobium japonicum*: use of *Rhizobium meliloti dct* gene(s) to enhance nitrogen fixation. J. Bacteriol. 170: 184-189.

de Bruijin, F.J., U. Hilgert, J. Stigter, M. Scheinder, H. Meyer, U. Klosse, and K. Pawlowski. 1990. Regulation of nitrogen fixation and assimilation genes in the free-living versus symbiotic state. p. 33-44. *In* P.M. Gresshoff, L. E. Roth, G. Stacey, and W.E. Newton (ed), Nitrogen fixation: achievements and objectives. Chapman and Hall Publishers, New York, USA.

Buchanan-Wollaston, V., M.C. Cannon, J.L. Beynon, and F.C. Cannon. 1981. Role of the *nifA* gene product in the regulation of *nif* expression in *Klebsiella pneumoniae.* Nature. 294: 776-778.

Cannon, F., J. Beynon, V. Buchanan-Wollaston, R. Burghoff, M. Cannon, R. Kwiatkowski, G. Lauer, and R. Rubin. 1985. Progress in understanding organization and expression of *nif* genes in *Klebsiella.* p453-460. *In* H.J. Evans, P.J. Bottomley, and W.E. Newton (ed.), Nitrogen fixation research progress. Martinus Nijhoff Publishers, Boston, USA.

Colonna-Romano, S., Arnold, W. A. Schlüter, P. Boistard, A. Pühler, And U.B. Priefer. 1990. An Fnr-like protein encoded in *Rhizobium leguminosarum* biovar *vicae* shows structural and functional homology to *Rhizobium meliloti* FixK. Mol. Gen. Genet. 223: 138-147.

Corbin, D., L. Barran, and G. Ditta. 1983. Organization and expression of *Rhizobium meliloti* nitrogen fixation genes. Proc. Natl. Acad. Sci. USA. 80: 3005-3009.

David, M., M-L. Daveran, J. Batut, A. Dedieu, O. Domergue, J Ghai, C. Hertig, P. Boistard, and D. Kahn. 1988. Cascade regulation of *nif* gene expression in *Rhizobium meliloti.* Cell. 54: 671-683.

David, M., O. Domergue, P. Pognonec, and D. Kahn. 1987. Transcription patterns of *Rhizobium melliloti* symbiotic plasmid pSym; identification of *nifA*- independent *fix* genes. J. Bacteriol. 169: 2239-2244.

Dénarié, J., F. Debelle and C. Rosenberg. 1992. Signalling and host range variation in nodulation. Annu. Rev. Microbiol. 46: 497-531.

Ditta, G.E. E. Virts, A. Palomares, and K. Choong-Hyun. 1987. The *nifA* gene of *Rhizobium meliloti* is oxygen regulated. J. Bacteriol. 169: 3217-3223.

Donald, R.G.K., D.W. Nees, C.K. Raymond, A.I. Loroch, and R.A. Ludwig. 1986. Characterization of three genomic loci encoding *Rhizobium* sp. strain ORS571 N_2 fixation genes. J. Bacteriol. 165: 72-81.

Earl, C.D., C.W. Ronson, and F.M. Ausubel. 1987. Genetic and structural analysis of the *Rhizobium meliloti fixA, fixB, FixC,* and *fixX* genes. J. Bacteriol. 169: 1127-1136.

el-Din, A.K. 1992. A succinate transport mutant of *Bradyrhizobium japonicum* forms ineffective nodules on soybeans. Can. J. Microbiol. 38: 230-234.

Fellay, R., J. Frey, and H. Krish. 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of gram-negative bacteria. Gene. 52: 147-154.

Finan, T.M., J.M. Wood, and D. Carlyle Jordan. 1983. Symbiotic properties of C_4-dicarboxylic acid transport mutants of *Rhizobium leguminosarum.* J. Bacteriol. 154: 1403-1413.

Fischer, H.M. 1994. Genetic regulation of nitrogen fixation in Rhizobia. Microbiol. Revs. 58: 352-386.

Fischer, H.M., T. Bruderer, and H. Hennecke. 1988. Essential and non-essential domains in the *Bradyrhizobium japonicum* NifA protein: identification of indespensible cysteine residues potetially involved in redox reactivity and/or metal binding. Nucleic Acids Res. 16: 2207-2224.

Fischer, H.M., G. Acuna, D. Anthamatten, F. Arigoni, M. Babst, P. Brouwer, T. Kaspar, I. Kullik, O. Preisig, B. Scherb, M. Weidenhaupt and H. Hennecke. 1993. Two oxygen-responsive regulatory cascades control nitrogen fixation genes in *Bradtrhizobium japonicum.* p. 411-416. *In* P. Palacios, J. Mora, and W.E. Newton (ed.), New horizons in nitrogen fixation. Kluwer Academic Publishers, Dordecht, The Netherlands.

Fuhrmann, M., H.M. Fischer, and H. Henncke. 1985. Mapping of *Rhizobium japonicum nifB-, fixBC-,* and *fixA*-like genes and identification of the *fixA* promoter. Mol. Gen. Genet. 199: 315-322.

Gabel, C., M.A. Bittinger, and R.J. Maier. 1994. Cytochrome aa3 gene regulation in members of the family Rhizobiaceae: comparison of copper and oxygen effects in *Bradyrhizobium japonicum* and *Rhizobium tropici.* Appl. Environ. Microbiol. 60: 141-148.

Gardiol, A.E., G.L. Truchet, and F.B. Dazzo. 1987. Requirement of succinate dehydrogenase activity for symbiotic bacteroid differentiation of *Rhizobium meliloti* in alfalfa nodules. Appl. Environ. Microbiol. 53: 1947-1950.

Gilles-Gonzalez, M.A., G. Ditta, and D. Helinski. 1991. A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti.* Nature. 350: 170-173.

Gilles-Gonzalez, M.A. and G. Gonzalez. 1993. Regulation of the kinase activity of hemeprotein FixL from the two-component system FixL/FixJ of *Rhizobium meliloti.* J. Bacteriol. 268: 16293-16297.

Gish, W. and D.J. States. 1993. Identification of protein coding regions by database similarity search. Nat. Genet. 3: 266-272.

Grönger, P., S.S. Manian, H. Reilander, M. O'Connell, U.B. Priefer, and A. Pühler. 1987. Orgsnisation and partial sequence of a DNA region of the *Rhizobium leguminosarum* symbiotic plasmid pRL6JI containing the genes *fixABC, nifA, nifB* and a novel open reading frame. Nucleic Acids Res. 15: 31-49.

Hardy, 1993. Biological nitrogen fertilization: present and future applications. p109-117. *In* Agriculture and environmental challenges. J.P. Srivastava and H. Aldermans (ed), Proceedings of the 13th Agric. Sector Symp. Washington D.C.: The World Bank.

Hennecke, H. 1990. Nitrogen fixation genes involved in the *Bradyrhizobium japonicum* soybean symbiosis. FEBS Lett. 268: 422-426.

Hennecke, H. 1993. The role of respiration in symbiotic nitrogen fixation. p. 55-64. *In* P. Palacios, J. Mora, and W.E. Newton (ed.), New horizons in nitrogen fixation. Kluwer Academic Publishers, Dordecht, The Netherlands.

Hennecke, H. A. Alvarez-Morales, M. Betancourt-Alvarez, S. Ebeling, M. Filser, H.M. Fischer, M. Gubler, M. Hahn, K. Kaluza, J.W. Lamb, L. Meyer, B. Regensburger, D. Studer, and J Weber. 1985. Organization and regulation of symbiotic nitrogen fixation genes from *Bradyrhizobium japonicum.* p157-163. *In* H.J. Evans, P.J. Bottomley, and W.E. Newton (ed.), Nitrogen fixation research progress. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

Hertig, C., R. Y. Li, A-M. Louarn, A-M. Garnerone, M. David, J. Batut, D. Kahn, and P. Boistard. 1989. *Rhizobium meliloti* regulatory gene *fixJ* activates transcription of *R. meliloti nifA* and *fixK* genes in *Escherichia coli.* J. Bacteriol. 171: 1736-1738.

Hirsch, A. M., C.A. Smith. 1987. Effects of *Rhizobium meliloti nif* and *fix* mutants on alfalfa root nodule development. J. Bacteriol. 169: 1137-1146.

Huala, E. and F.M. Ausubel. 1989. The central domain of *Rhizobium meliloti* NifA is sufficient to activate transcription from the *R. meliloti nifH* promoter. J.Bacteriol. 171: 3354-3365.

Hynes, M.F. and N. McGregor. 1990. Two plasmids other than the nodulation plasmids are neccesary for the formation of nitrogen-fixing nodules by *Rhizobium leguminosarum.* Mol. Microbiol. 4: 567-574.

Hynes, M.F., J. Quandt, A Schlüter, T Paschkowski, S. Weidner, and U.B. Priefer. 1992. Abstr. Book ninth int congr. nitrogen fixation, abstr. 464.

Iismaa, S.E., P.M. Ealing, K.F. Scott, and J.M. Watson. 1989. Molecular linkage of the *nif/fix* and *nod* gene regions in *Rhizobium leguminosarum* biovar *trifolii.* Mol. Microbiol. 3: 1753-1764.

Iismaa, S.E. and J.M. Watson. 1989. The *nifA* gene product from *Rhizobium leguminosarum* biovar *trifolii* lacks the N-terminal domain found in other NifA proteins. Mol. Microbiol. 3: 943-955.

Innes, R.W., M. Hirose, and P.L. Kuempel. 1988. Introduction of nitrogen-fixing nodules on clover requires only 32kilobase pairs of DNA from the *Rhizobium trifolii* symbiosis plasmid. J. Bacteriol. 170: 3793-3802.

Kahn, D., M. David, O. Domergue, M-L. Daveran, J. Ghai, P.R. Hirsch, and J. Batut. 1989. *Rhizobium meliloti fixGHI* sequence predicts involvement of a specific cation pump in symbiotic nitrogen fixation. J. Bacteriol. 171: 929-939.

Kahn, D., and G. Ditta. 1991. Modular structure of FixJ: homology of the transcriptional activator domain with the -35 binding domain of sigma foctors. Mol. Microbiol. 5: 987-997.

Kaminski, P.A. and C. Elmerich. 1991. Involvement of *fixLJ* in the regulation of nitrogen fixation in *Azorhizobium caulinodans.* Mol. Microbiol. 5: 1983-1991.

Kaminski. P.A., K. Mandon, F. Arigoni, N. Desnoues, and C. Elmerich. 1991. Regulation of nitrogen fixation in *Azorhizobium caulinodans*: identification of a *fixK*-like gene, a positive regulator of *nifA*. Mol. Microbiol. 5: 1983-1991.

Kaminski, P.A., F. Norel, N. Desnoues, A. Kush, G. Salzano, and C. Elmerich. 1988. Characterizations of the *fixABC* region of *Azorhizobium caulinodans* ORS571 and identification of a new nitogen fixation gene. Mol. Gen. Genet. 214: 496-502.

Kanamura, K., S. Kashiwagi, T. Mizuno. 1994. A copper-transporting P-type ATPase found in the thylakoid membrane of the

cyanobacterium *Synechococcus* species PCC7942. Mol. Microbiol. 13: 369-377.

Kolb, A., S. Busby, H. Buc, S. Garges, and S. Adhya. 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. 62: 749-795.

Labes, M., V. Rastogi, R. Watson, and T.M. Finan. 1993. Symbiotic nitrogen fixation by a *nifA* deletion mutant of *Rhizobium meliloti*:: the role of an unusual *ntrC* allele. J. Bacteriol. 175: 2662-2673.

Lois, A.F., G. Ditta, and D. Helinski. 1993a. The oxygen sensor FixL of *Rhizobium meliloti* is a membrane protein containing four possible transmembrane segments. J. Bacteriol. 175: 1103-1109.

Lois, A.F., M. Weinstein, G. Ditta, and D. Helinski. 1993b. Autophosphorylation and phosphatase activities of the oxygen sesing proteinFixL of*Rhizobium meliloti* are coordinately regulated by oxygen. J. Biol. Chem. 268: 4370-4375.

Ludden, P.W. 1993. *nif* gene products and their roles in nitrogen fixation. p. 101-104. *In* P. Palacios, J. Mora, and W.E. Newton (ed.), New horizons in nitrogen fixation. Kluwer Academic Publishers, Dordecht, The Netherlands.

de Maagd, R.A., W.C. Yang, L. Goosen-de Roo, I.H.M. Mulders, H.P. Roest, H.P. Spaink, T. Bisseling, and B.J.J. Lugtenberg. 1994. Down-regulation of expression of the *Rhizobium leguminosarum* outer membrane protein gene *rpoA* occurs abruptly in interzone II-III of pea nodules and can be uncoupled from *nif* gene activation. Mol. Plant. Microbe. Interact. 7: 276-281.

Mahl, M.C., P. W. Wilson, M.A Fife, and W.H. Ewing. 1965. Nitrogen fixation by members of the tribe *Klebsielleae*. J. Bacteriol. 89: 1482-1487.

Mandon, K., P. Alexandre Kaminski, and C. Elmerich. 1994. Functional analysis of the *fixNOQP* region of *Azorhizobium caulinodans*. J. Bacteriol. 176: 2560-2568.

Mandon, K., P. A.lexandre Kaminski, C. Mougel, N. Desnoues, B. Dreyfus, and C. Elmerich. 1993. Role of the *fixGHI* region of

Azorhizobium caulinodans in free-living and symbiotic nitogen fixation. FEMS Microbiol. lett. 114: 185-190.

Mavridou, A., M.A. Barny, P. Poole, K. Plaskitt, A.E. Davies, A.W. Johnston, and J.A. Downie. 1995. *Rhizobium leguminosarum* nodulation gene (*nod*) expression is lowered by an allele-specific mutation in the dicarboxylate transport gene *dctB*. Microbiology. 141: 103-111.

Meade, H.M., S.R. Long, G.B. Ruvken, S.E. Brown, and F.M. Ausubel. 1982. Physical and genetic characterization of symbiotic and auxotrophic *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. J. Bacteriol. 149: 114-122.

Merrick, M.J. 1993. Organization and regulation of nitrogen fixation genes. p. 43-54. *In* P. Palacios, J. Mora, and W.E. Newton (ed.), New horizons in nitrogen fixation. Kluwer Academic Publishers, Dordecht, The Netherlands.

Monson, E.K., G. Ditta, and D. Helinski. 1995. The oxygen sensor protein, FixL, of *Rhizobium meliloti.* J. Biol. Chem. 270: 5243-5250.

Monson, E.K., A.F Lois, P.G. Agron, M. Weinstein, S.W. Stanfield, G.S Ditta, and D.R. Helinski. 1993. Oxygen sensing by the *Rhizobium meliloti* two -component regulatory system FixLJ. p. 405-410. *In* P. Palacios, J. Mora, and W.E. Newton (ed.), New horizons in nitrogen fixation. Kluwer Academic Publishers, Dordecht, The Netherlands.

Monson, E.K., M. Weinstein, G. Ditta, and D. Helinski. 1992. The FixL of *Rhizobium meliloti* can be seperated into a heme-binding oxygen sensing domain and a functional C-terminal kinase domain. Proc. Natl. Acad. Sci. USA. 89: 4280-4284.

Morett, E. and M. Buck. 1988. NifA-dependent *in vivo* protection demonstrates that the upstream activator sequence of *nif* promoters is a protein binding site. PProc. Natl. Acad. Sci. USA. 85: 9401-9405.

Morett, E., H-M. Fischer, and H. Hennecke. 1991. Influence on DNA binding, positive control, and stability of the *Bradyrhizobium japonicum* NifA regulatory protein. J. Bacteriol. 173: 3478-3487.

National research council, Board of science and technology for international development. 1994. Biological nitrogen fixation, research challenges. National Academy Press. Washington D.C. USA.

Nees, D.W., P.A. Stein, and R.A. Ludwig. 1988. The *Azorhizobium caulinodans nifA* gene: identification of upstream-activating sequences including a new element, the anaerobox. Nucleic Acids Res. 16: 9839-9853.

Neidle, E.L. and S. Kaplan. 1992. *Rhodobacter sphaeroides rdxA*, a homolog of *Rhizobium meliloti fixG,* Encodes a membrane protein which may bind cytoplasmic [4Fe-4S] clusters. J. Bacteriol. 174: 6444-6454.

Nixon, B.T., C. W. Ronson, and F. M. Ausubel. 1986. Two component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes *ntrB* and *ntrc.* Proc. Natl. Acad. Sci. USA 83: 7850-7854.

Noonan, B., M. Motherway, and F. O'Gara. 1992. Ammonia regulation of the *Rhizobium meliloti* nitrogenase structural and regulatory genes under free-living conditions: involvement of the FixL gene product. Mol. Gen. Genet. 234: 423-428.

O'Connell. 1984. Genetic transfer in prokaryotes: transformation, transduction, and onjugation. p. 9-12. *in* A. Pühler and K.N. Timmis (eds). Advanced molecular genetics. Springer-Verlag, Berlin, Germany.

Odermatt, A., H. Suter, R. Krapf, and M. Solioz. 1993. Primary structure of two P-type ATPases involved in copper homeostasis in *Enterococcus hirae.* J. Biol. Chem. 268: 12775-12779.

Page, K.M. and M.L. Guerinot. 1995. Oxygen control of the *Bradyrhizobium japonicum hemA* gene. J. Bacteriol. 177: 3979-3984.

Pawlowski, K., P. Ratet, J. Schell, and F.J. de Bruijin. 1987. Cloning and characterization of *nifA* and *ntrC* genes of the stem nodulating bacterium ORS571, the nitrogen fixing symbiont of *Sesbania*

rostrata: Regulation of nitrogen fixation (*nif*) genes in the free living versus symbiotic state. Mol. Gen. Genet. 206: 207-219.

de Phiip, P., J. Batut, and P.Boistard. 1990. *Rhizobium meliloti* FixL is an oxygen sensor and regulates *R. meliloti nifA* and *fixK* genes differently in *Escherichia coli.* J. Bacteriol. 172: 4255-4262.

Phung, L.T., G. Ajilani, and R. Haselkorn. 1994. P-type ATPase from the cyanobacterium *Synechococcus* 7942 related to the human Menkes and Wilson disease gene products. Proc. Natl. Acad. Sci. USA. 91: 9651-9654.

Pridmore, R.D. 1987. New and versatile cloning vectors with kanamycin resistance marker. Gene. 56: 309-312.

Priefer, U.B. 1989. Genes involved in lipopolysaccharide production and symbiosis are clustered on the chromosome of *Rhizobium leguminosarum* biovar *viciae* VF39. J. Bacteriol. 171: 6161-6168.

Preisig, O., D. Anthamatten, and H. Hennecke. 1993. Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen fixing edosymbiosis. Proc. Natl. Acad. Sci. USA. 90: 3309-3313.

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

Rastogi, V., M. Labes, T. Finan, and R. Watson. 1992. Overexpression of the *dctA* gene in *Rhizobium meliloti:* effect on transport of C4 dicarboxylates and symbiotic nitrogen fixation. Can. J. Microbiol. 38: 555-562.

Ratet, P., K. Pawlowski, J. Schell, and F.J. de Brujin. 1989. The *Azorhizobium caulinodans* nitrogen-fixation regulatory gene, *nifA,* is controlled by the cellular nitrogen and oxygen status. Mol. Microbiol. 3: 825-838.

Raven , P.H., R. Evert, and S. Eichorn. 1986. Biology of plants, Worth, New York. 517-541.

Renalier, M.H., J Batut J. Ghai, B. Terzaghi, M. Ghérardi, M. David, A-M. Garnerone, J Vasse, G. Truchet, T. Huguet, and P. Boistard. 1987. A new symbiotic cluster of the pSym megaplasmid of *Rhizobium meliloti* 2011 carries a functional *fix* gene repeat and a *nod* locus. J. Bacteriol. 169: 2231-2238.

Reyrat, J-M., M. David, C. Blonski, P. Boistard, J. Batut. 1993. Oxygen-regulated in vitro transcription of *Rhizobium meliloti nifA* and *fixK* genes. J. Bacteriol. 175: 6867-6872.

Reyrat, J-M. M. David, J Batut, P. Boistard. 1994. FixL of *Rhizobium meliloti* enhances the transcriptional activity of a mutant FixJD54N protein by phosphorylation of an alternate residue. J. Bacteriol. 176: 1969-1976.

Ronson, C.W., P.M. Astwood, and J.A. Downie. 1984. Molecular cloning and genetic organization of C4-dicarboxylate transport genes from *Rhizobium leguminosarum.* J. Bacteriol. 160: 903-909.

Ronson, C.W., P. Lyttleton, and J.G. Robertson. 1981. C4-dicarboxylate transport mutants of *Rhizobium trifolii* form ineffective nodules on *Trifolium repens.* Proc. Natl. Acad. Sci. USA. 78: 4284-4288.

Ruvkun, G.B., V. Sundaresan, and F.M. Ausubel. 1982. Directed transposon Tn5 mutagenesis and complementaion analysis of *Rhizobium meliloti* symbiotic nitrogen fixation genes. Cell. 29: 551-559.

Sambrook, J., E.F. Fritsch, and T. Maniatus. 1989. Molecular cloning, a laboratory manual. Cold spring Harbor New, York.

Santero, E., T. Hoover, J. Keener, and S. Kustu. 1989. *In vitro* activity of the nitogen fixation regulatory protein NifA. Proc. Natl. Acad. Sci. USA. 86: 7346-7350.

Schlüter, A., T. Patschokwski, G. Unden, and U.B. Priefer. 1992. The *Rhizobium leguminosarum* FnrN protein is functionally similar to *Escheria coli* Fnr and promotes heterologous oxygen-dependent activation of transcription. Mol. Microbiol. 6: 3395-3404.

Schlüter, A., S. Ruberg, M. Kramer, S. Weidner, and U.B. Priefer. 1995. A homolog of the *Rhizobium meliloti* nitrogen fixation gene *fixN* is involved in the production of a microaerobically induced oxidase activity in the phytopathogenic bacterium *Agrobacterium tumefaciens.* Mol. Gen. Genet. 247: 206-215.

Schweizer, H.P. 1993. Two plasmids, X1918 and Z1918, for easy recovery of the *xylE* and *lacZ* reporter genes. Gene. 134: 89-91.

Shah, V.K., G. Stacey, and W.J. Brill. 1983. Electron transport to nitrogenase. J. Biol. Chem. 258: 12064-12068.

Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotech. 1: 784-791.

Soupene, E., M. Foussard, P. Boistard, G. Truchet, and J. Batut. 1995. Oxygen as a key developmental regulator of *Rhizobium meliloti* N_2 fixation gene expression within the alfalfa root nodule. Proc. Natl. Acad. Sci. USA. 92: 3759-3763.

Szeto, W.W., J.L. Zimmerman, V. Sundaresan, F.M. Ausubel. 1984. A *Rhizobium meliloti* symbiotic regulatory gene. Cell. 36: 1035-1043.

Thöny, B., D. Anthamatten, T. Bruderer, and H. Hennecke. 1987. The symbiotic nitrogen fixation regulatory operon(*fixRnifA*) of *Bradyrhizobium japonicum* is expressed aerobically and is subject to a novel, *nifA*-independent type of activation. Nucleic Acids Res. 15: 8479-8499.

Thöny-Meyer, L., C. Beck, O. Preisig, and H. Hennecke. 1994. The *ccoNOQP* gene cluster codes for a cb-type cytochrome oxidase that functions in aerobic respiration of *Rhodaobacter capsulatus.* Mol. Microbiol. 14: 705-716.

Urban, J.E., and F. Dazzo. 1982. Succinate-induced morphology of *Rhizobium trifolii* 0403 resembles that of bacteroids in clover nodules. Appl. Environ. Microbiol. 44: 219-226.

Vasse, J., F. de Billy, S. Camut, and G. Truchet. 1990. Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J. Bacteriol. 172: 4295-4306.

Waelkens, F., A. Foglia, J-B Morel, J. Fourment, J. Batut, and P. Boistard. 1992. Molecular genetic analysis of the *Rhizobium meliloti fixK* promoter: identification of sequences involved in positive and negative regulation. Mol. Microbiol. 6: 1447-1456.

Watson, R. J., Y.K. Chan, R. Wheatcroft, A.F. Yang, and S. Han. 1988. *Rhizobium meliloti* genes required for C_4-dicarboxylate transport and symbiotic nitrogen fixation are located on a megaplasmid. J. Bacteriol. 170: 927-934.

Weinstein, M., A.F. Lois, E.K. Monson, G. Ditta, and D. Helinski. 1992. Isolation of phosphorylation-deficient mutants of the *Rhizobium meliloti* two-component regulatory protein, FixJ. Mol. Microbiol. 6: 2041-2049.

Werner, D. 1992. Physiology of nitrogen-fixing legume nodules: compartments and functions. p. 399-431. *In* G. Stacey, R.H. Burris, H. J. Evans (ed), Biological nitrogen fixation. Chapman and Hall Publishers, New York, USA.

Wootton, J.C. and M. Drummond. 1989. The Q-linker: a class of interdomain sequences found in bacterial multidomain regulatory proteins. Protein Eng. 2: 535-534.

Zimmerman, J.L., W.W. Szeto, and F.M. Ausubel. 1983. Molecular characterization of Tn5-induced symbiotic (Fix-) mutants of *Rhizobium meliloti.* J. Bacteriol. 156: 1025-1034.

Appendix 1. Medium and commonly used solutions

1 Medium

1.1 LB

Tryptone	10.0g
Yeast extract	5.0g
NaCl	10.0g
H2O	1.0L

(12.5g agar for solid media)

1.2 PA

Antibiotic broth	17.5g
H2O	1.0L

1.3 TB

KH2PO4	2.32g
K2HPO4	12.54g
Tryptone	12.0g
Yeast extract	24.0g
Glycerol	4.0ml
H2O	1.0L

1.4 TY

Tryptone	5.0g
Yeast extract	3.0g
CaCl2	0.5g
H2O	1.0L

1.5 PH

Peptone	4.0g
Yeast extract	0.5g
Tryptone	0.5g
CaCl2	0.2g
MgSO4	0.2g
H20	1.0L

1.6 VMM

1.6.1 Solution A

K2HPO4	1.0g
KH2PO4	1.0g
H2O	1.0L

1.6.2 Solution B

FeCl3	0.1g
MgSO4	2.5g
CaCl2	1.0g
H2O	1.0L

1.6.3 Solution C

Biotin	0.01g
Thiamine	0.01g
Ca Pantathenate	0.01g
H2O	1.0L

1.7 Tris borate bufffer

Tris base	216.0g
Boric acid	110.0g
EDTA	14.88g
H2O	20L

1.8 Plant medium

1.8.1 Solution A

CaCl	294.0g
H2O	1.0L

1.8.2 Solution B

KH2PO4	136.0g
H20	1.0L

1.8.3 Solution C

Fe-citrate	6.7g
H20	1.0L

1.8.4 Solution D

MgSO4	123.0g
K2SO4	87.0g
MnSO4	0.338g
H2BO4	0.247g
ZnSO4	0.288g
CuSO4	0.1g
CoSo	0.056g
Na2MoO4	0.048g
H2O	1.0L

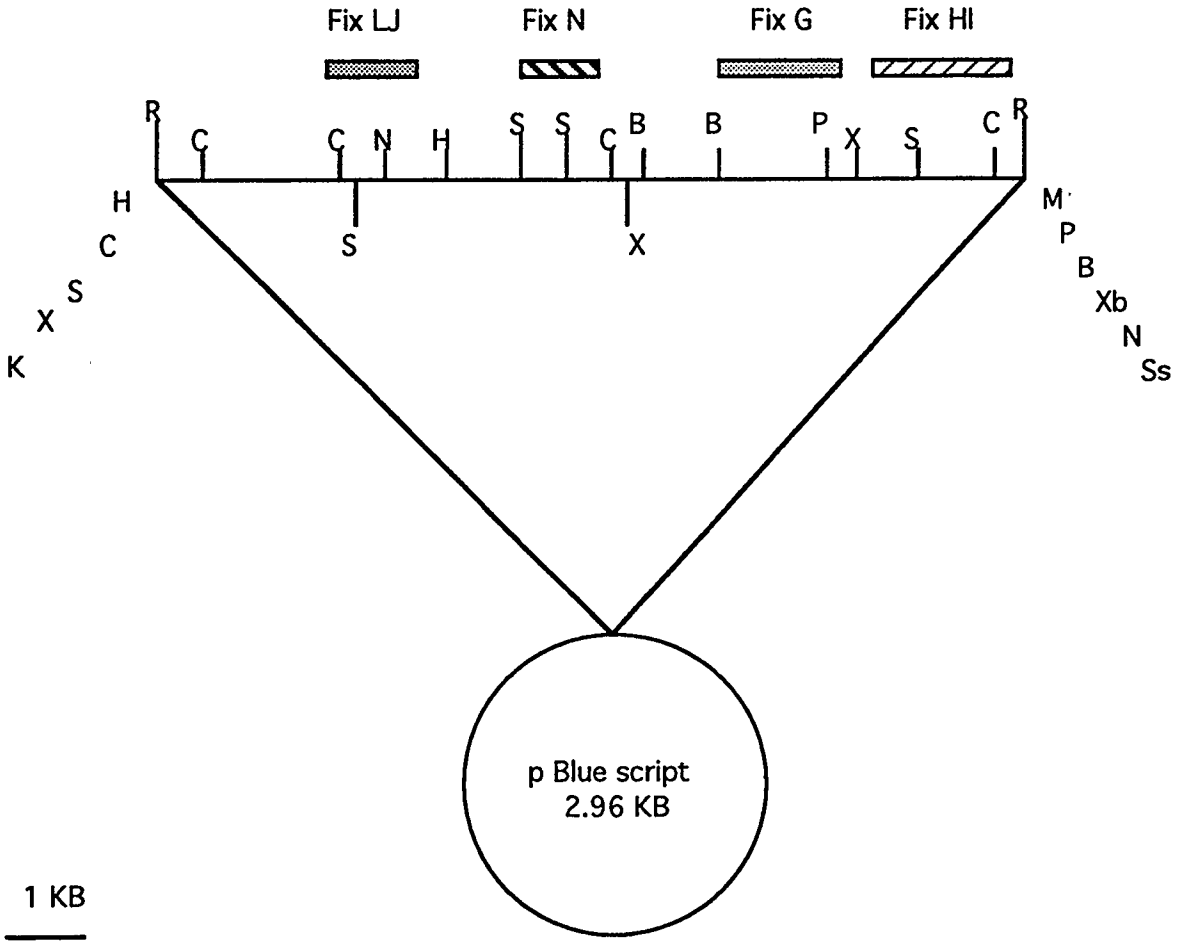
1.0ml of each of these solutions is added to 2.0L of dH2O

1.9 Total DNA preparations

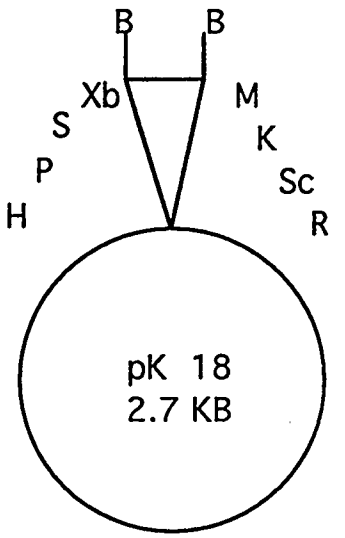
-1.5ml of late log cuulture into 1.5ml eppendorf tube. Spin for 1 minute at 13000rpm.

- Resuspend pellet in 1ml 1M NaCl; leave 1hour on ice.
- 3 minutes at 13000rpm; decant supernatant.
- Resuspend pellet in 250microlitres of 20% sucrose in TE buffer.
- Add 250microlitres of lysozyme (5mg/ml)/RNase (1mg/ml) in TE; vortex and incubate for 30 minutes at 37c.
- Add 100microlitres of 5% sarcosyl/ProteinaseK (5mg/ml) in TE, mix and leave overnight at 37c.
- Add 70microlitres of 3M Na-acetate pH 5.4, 300microlitres phenol, and 100microlitres chloroform; mix gently for 2-5 minutes.
- 10 minutes at 13000rpm, remove aqueous phase.
- Add 100microlitres phenol and 100microlitres chloroform, mix gently for 1 minute, spin at 13000rpm for 10 minutes. Remove aqueous phase.
- Add 700microlitres isopropanol, mix and leave in -80c freezer for 1 hour.
- Spin at 13000rpm for 30 minutes, decant supernatant, rinse pellet in 70% ethanol twice. Remove 70% ethanol and dry under vaccum for 5 minutes.
- Resuspend pellet in 10microlitres sterile distilled water, leave at 65c for 30 minutes and store at 4^{o}c for 2-3 days before use.

Appendix 2. Plasmids produced

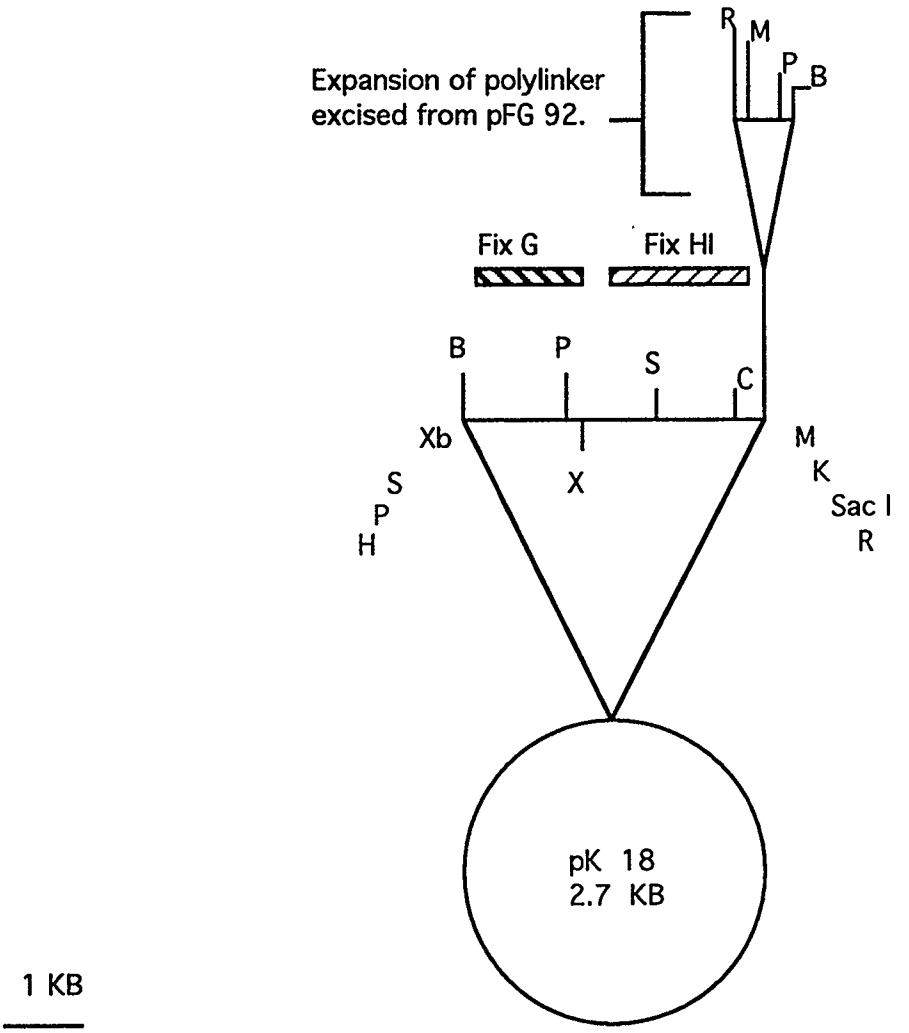


Date: Sept 11/92.
Name: pFG 92
Previous titles: 3-3, 3-1.
Source: C plasmid of R. leguminosarum.
Notes: Isolated from cosmid library created by Eco RI digest of plasmid and host DNA. Several (2) Bgl I sites from the left Eco RI site to the first Bam HI site were found.

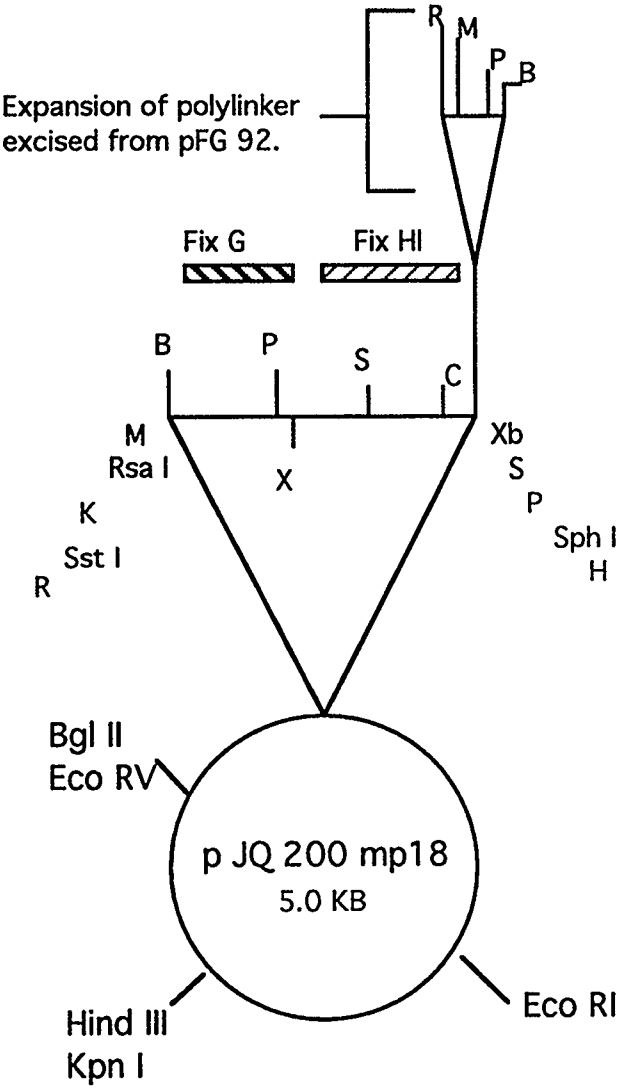


1 KB

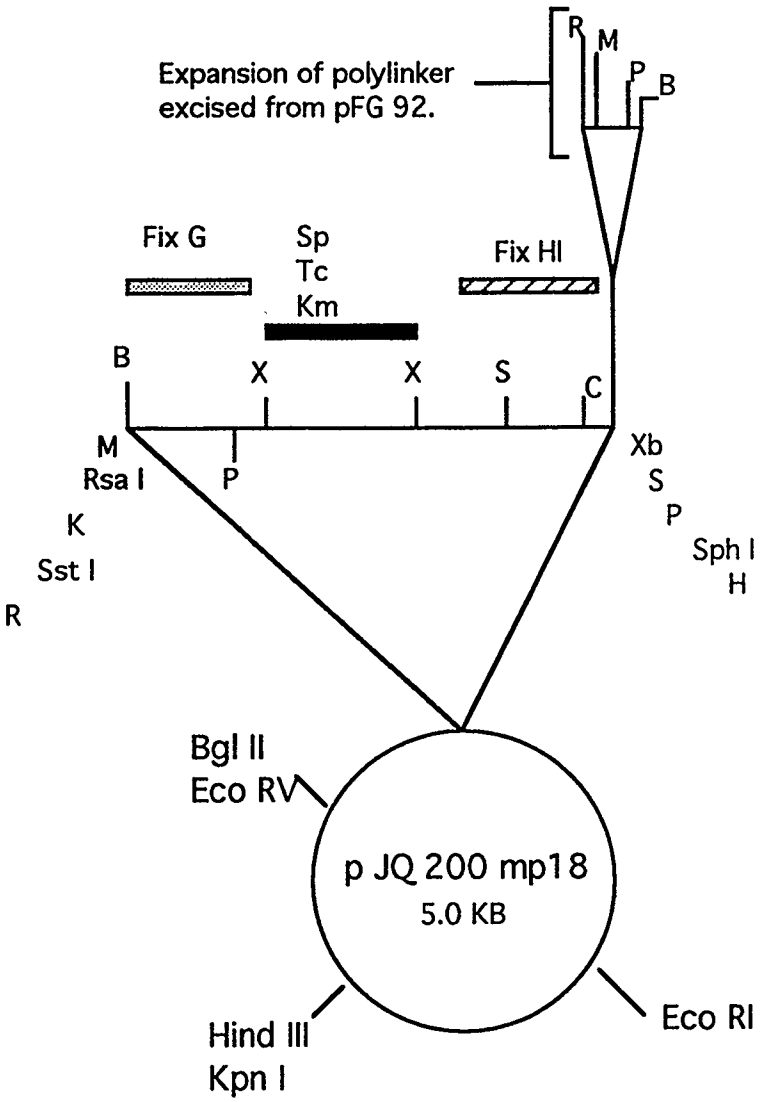
Date: Oct 27/92
Name: pFG 92-1.
Previous titles: B2.
Source: Subclone of pFG 92.
Notes: Created by Bam HI digest of pFG92.



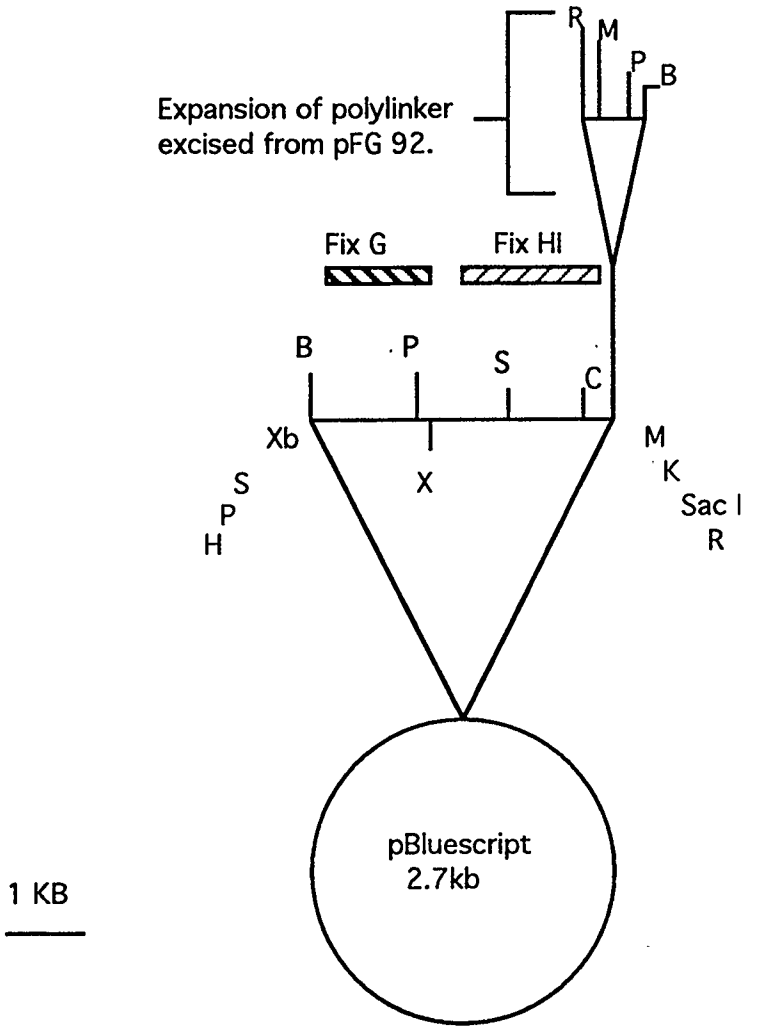
Date: Oct 27/92.
Name: pFG 92-4.
Previous titles: B3, B4, B7, B11, B17.
Source: Subclone of pFG 92.
Notes: Created by Bam HI digest of pFG92.
pFG 92-4 rev has the Bam HI segment
in the opposite orientation.



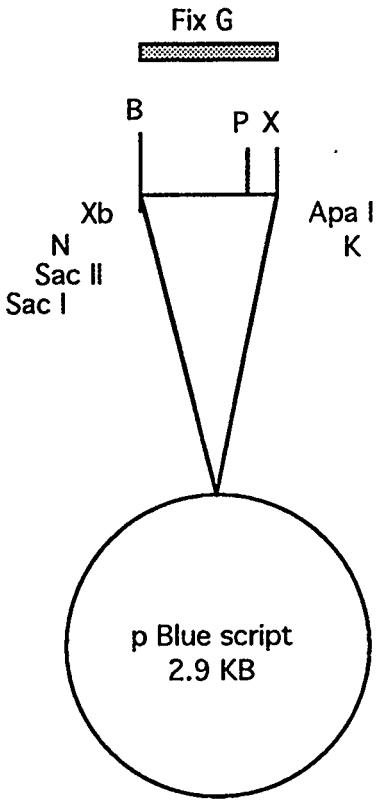
Date: Jan 9/93.
Name: pFG 92-4 mp 18.
Previous titles: 8.
Source: Subclone of pFG 92-4 .
Notes: Created by Bam HI digest of pFG92-4 and Bam HI digest of pJQ 200mp 18. Reverse orientation exists and designated pFG 92-4 mp 18 rev.



Date: Jan 18/93.
Name: pFG 92-4 mp 18 Sp, Km, or Tc.
Previous titles: Tc1,2,3, Sp1, Km 1,6.
Source: Subclone of pFG 92-4 mp 18.
Notes: Created by Xho I digest of pFG 92-4 mp 18, endfilling and ligating it to the Sma I fragments of the interposons. Orientation of interposon resistance marker is unknown.

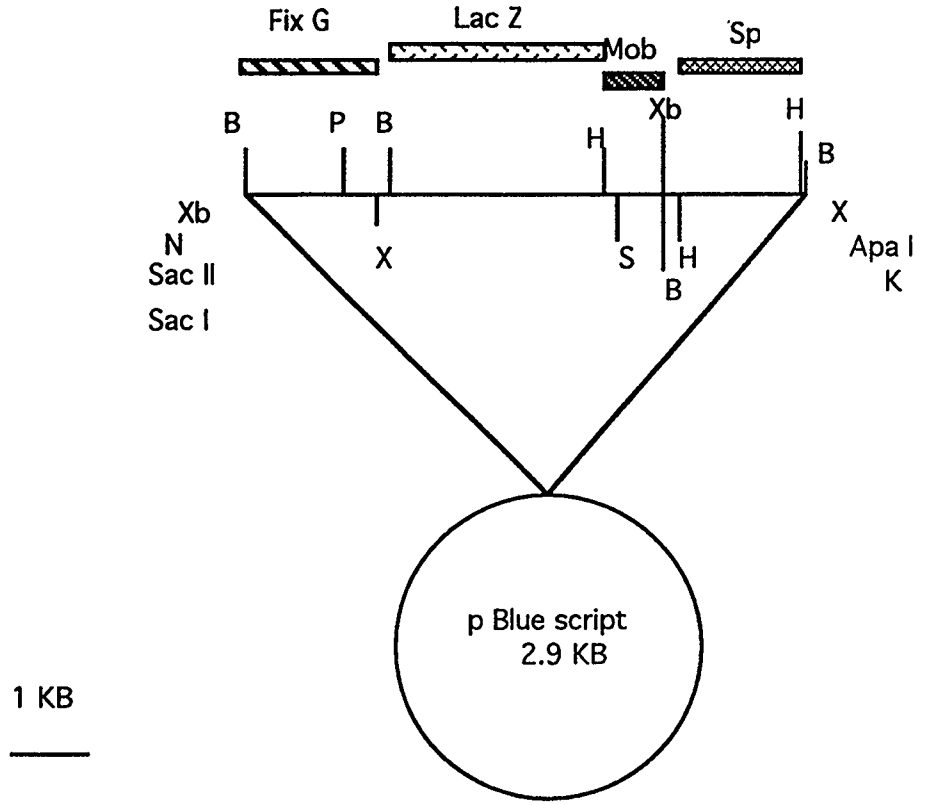


Date: April 25/95.
Name: pFG 92-4Blu
Previous titles: 92i
Source: Subclone of pFG 92-4
Notes: Created by Bam HI digest of pFG92-4
and ligation of fragment to pBluescript.

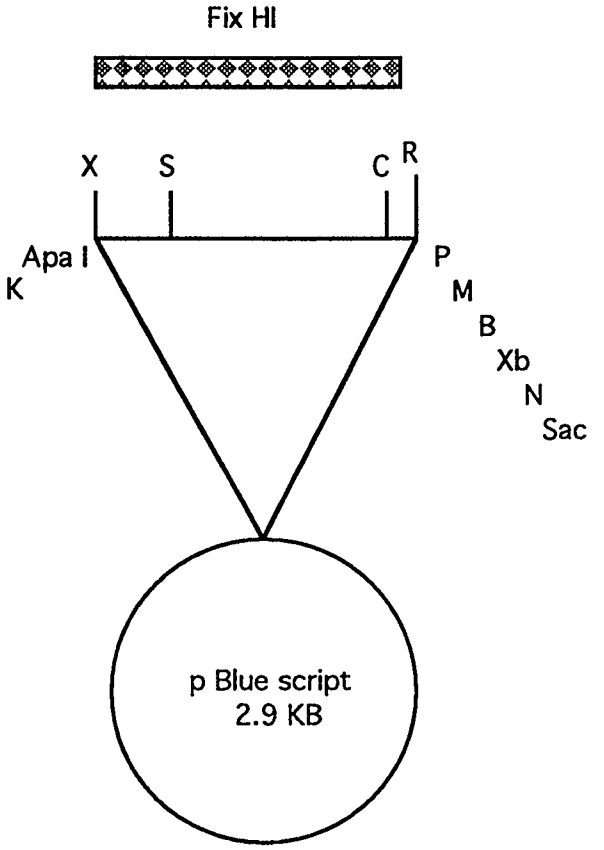


1 KB

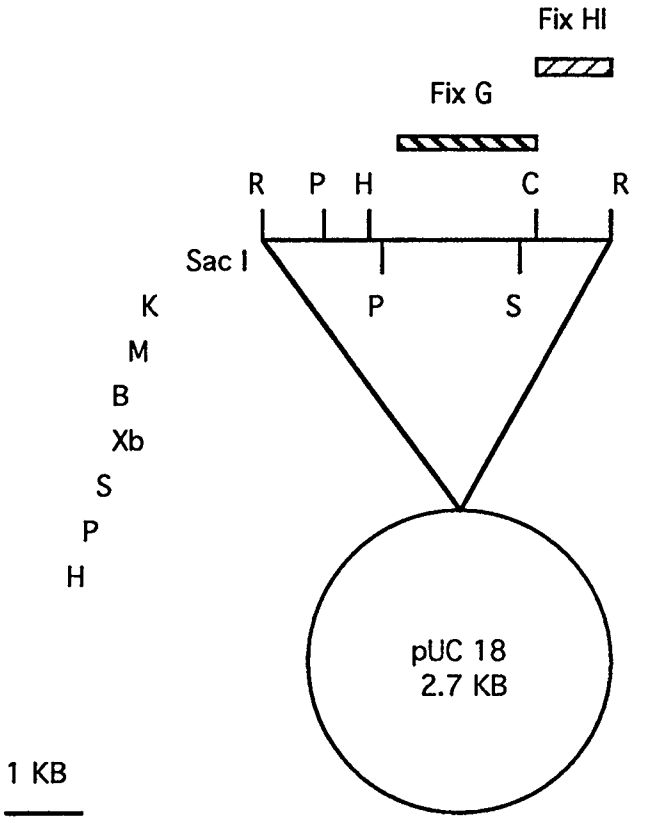
Date : Dec 17/92.
Name: pFG 92-4a
Previous titles: 4a-3, 4a-5, 4a-7, 4a-8.
Source: Subclone of pFG 92-4.
Notes: Created by Xho/Eco RI/Xba digest of pFG 92-4 and ligation with p Blue script SK digested with Xba I and Xho I.



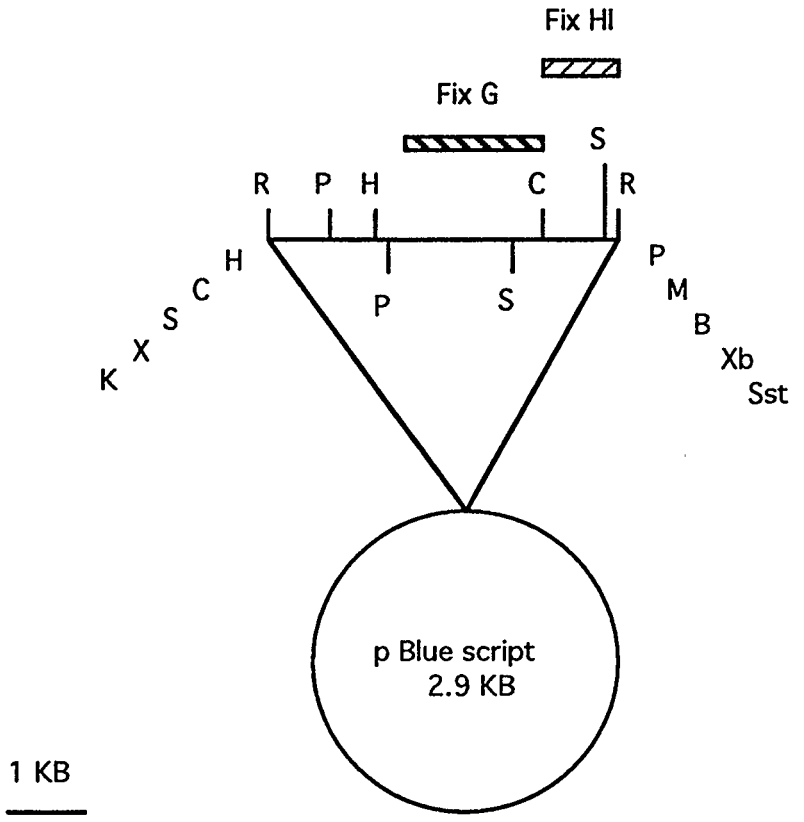
Date: Jan 1/93.
Name: pFG 92-4a lac.
Previous titles: Sample 3.
Source: Product of pFG 92-4a.
Notes: Created by digesting pFG 92-4a with Xho I, endfilling, and ligating it to the Sma I fragment of the lac cassette. Xho I sites retained at termini of Fix G and Sp gene.



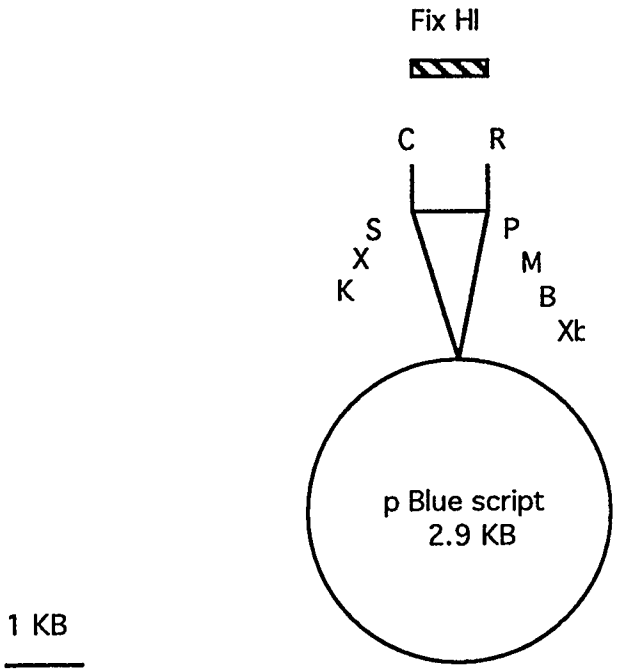
Date: Dec 17/92
Name: pFG 92-4b
Previous titles: 4b-1, 4b-3, 4b-4, 4b-5
Source: Subclone of pFG 92-4.
Notes: Created by Xho I/Eco RI/Xba I digest of pFG 92-4 and ligation with pBlue script digested with XhoI and Eco RI.



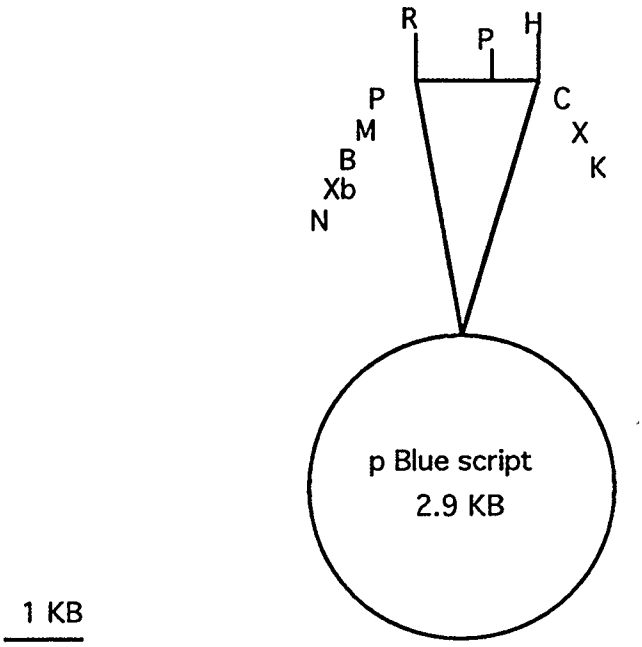
Date : Sept 12/92.
Name: pFG 93
Previous titles: 4-1.
Source: D plasmid of R. leguminosarum.
Notes: Isolated from cosmid library created by Eco RI digest of plasmid and host DNA.



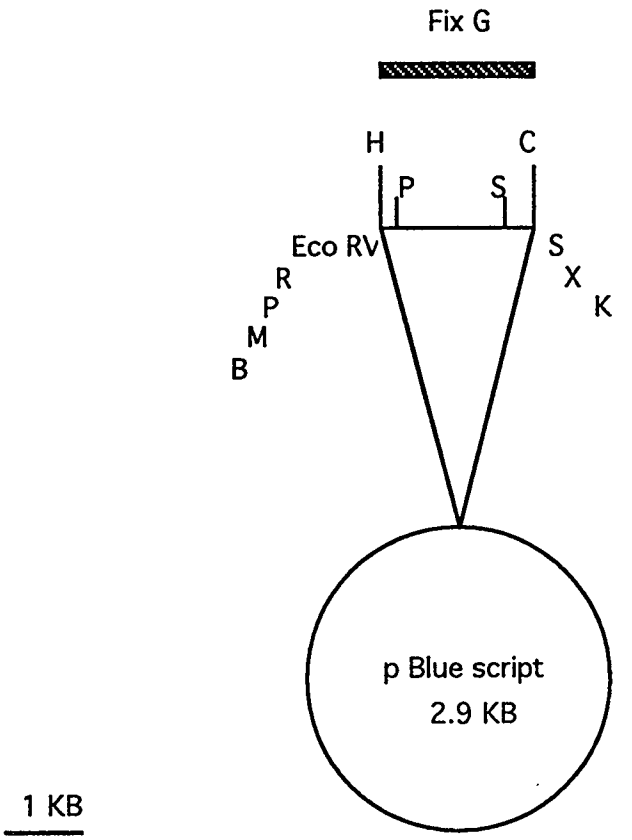
Date : June 7/93.
Name: pFG 93 Blu
Previous Titles: 19
Source: Derivative of pFG 93.
Notes: Created by digestion of pFG 93 with Eco RI, gene cleaning the fragment and ligating it into p Blue script. Reverse orientation also produced and designated pFG 93 Blu rev.



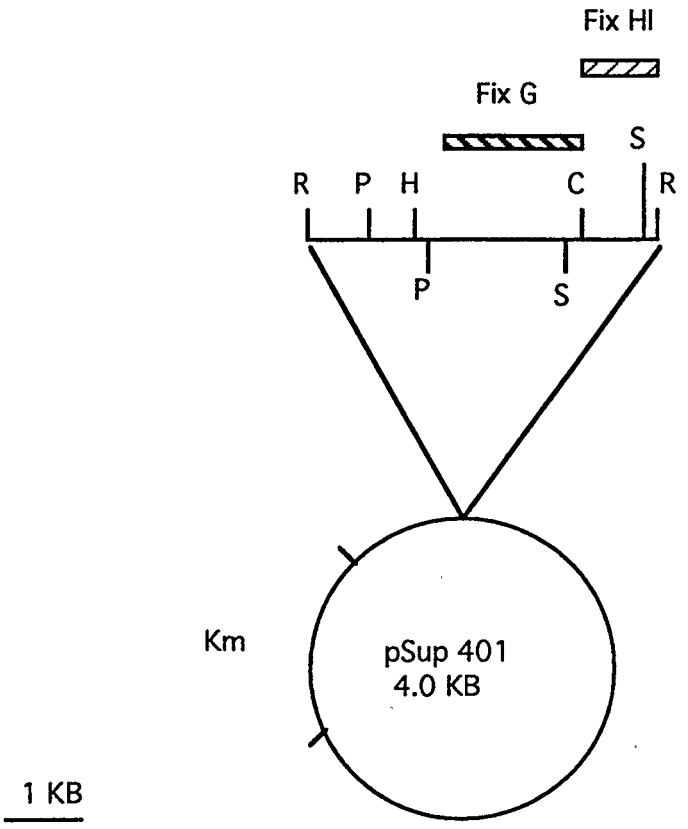
Date: Nov 24/92.
Name: pFG 93-1.
Previous titles: None.
Source: Subclone of pFG 93.
Notes: Created by Eco RI/Cla digest of pFG 93.



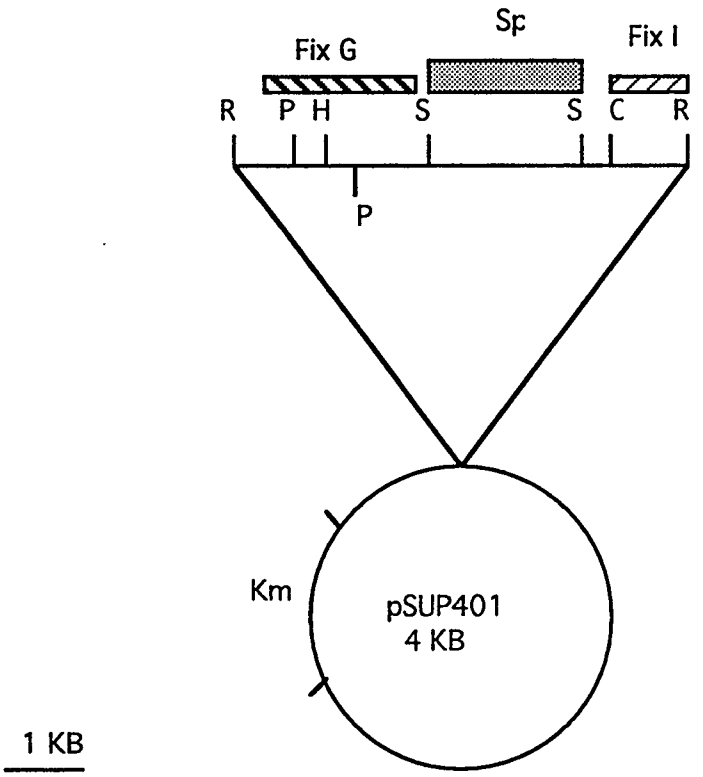
Date Nov 24/92
Name: pFG 93-2
Previous titles:
Source: subclone of pFG 93.
Notes: Produced by an Eco RI/Hind III digest.



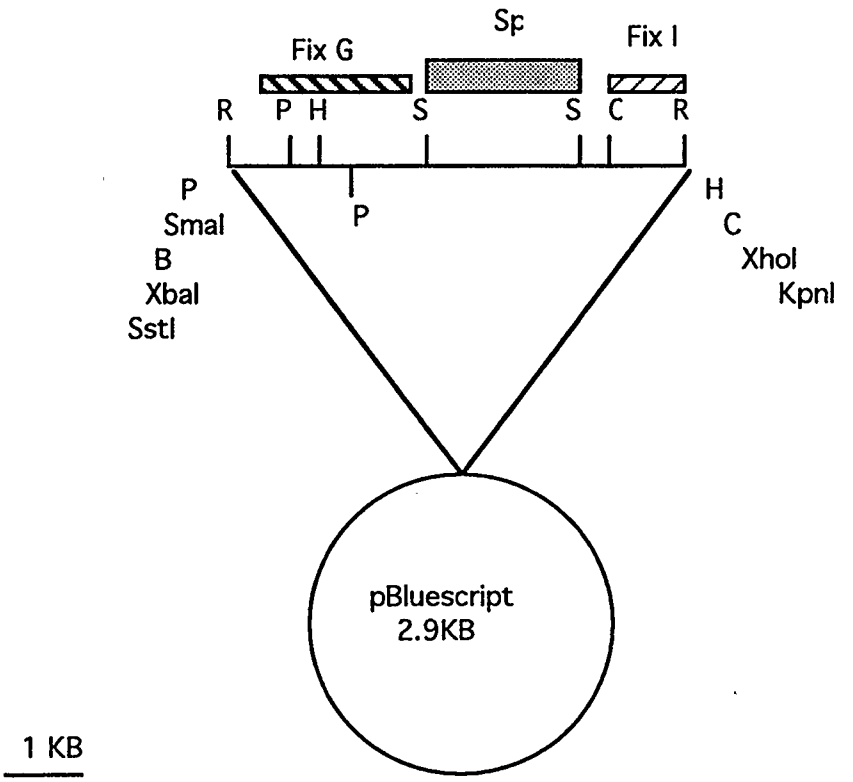
Date: Nov 24/92.
Name: pFG 93-3.
Previous titles: None.
Source: Subclone of pFG 93.
Notes: Cloned by Hind III/Cla I digest. Contains Fix G gene of the D plasmid.



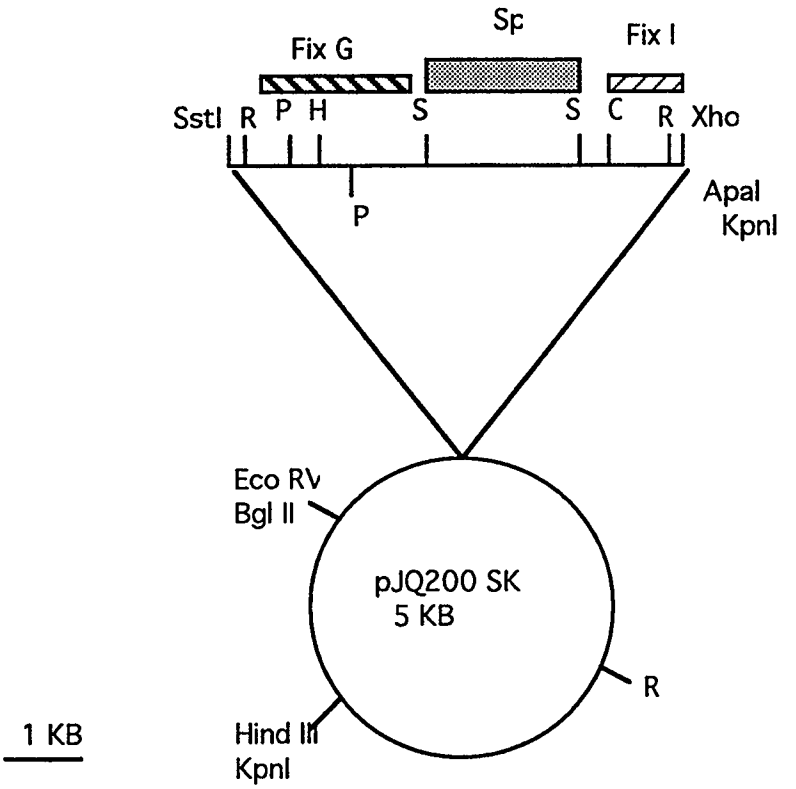
Date: Dec 23/93
Name: pSUP401-93
Previous titles: None
Source: pFG 93
Notes: EcoRI fragment inserted into pSUP401 inactivating Cm gene.



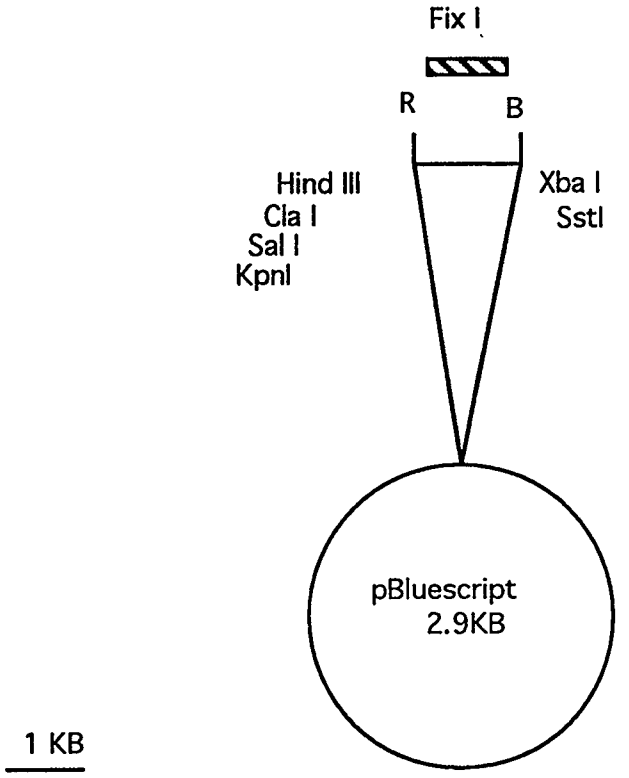
Date: Mar 5/94
Name: pSUP401-93Sp
Previous titles: Ks12
Source: pSUP401-93
Notes: Produced from pSUP401-93 by inserting a Sp cassette at the unique SalI site.



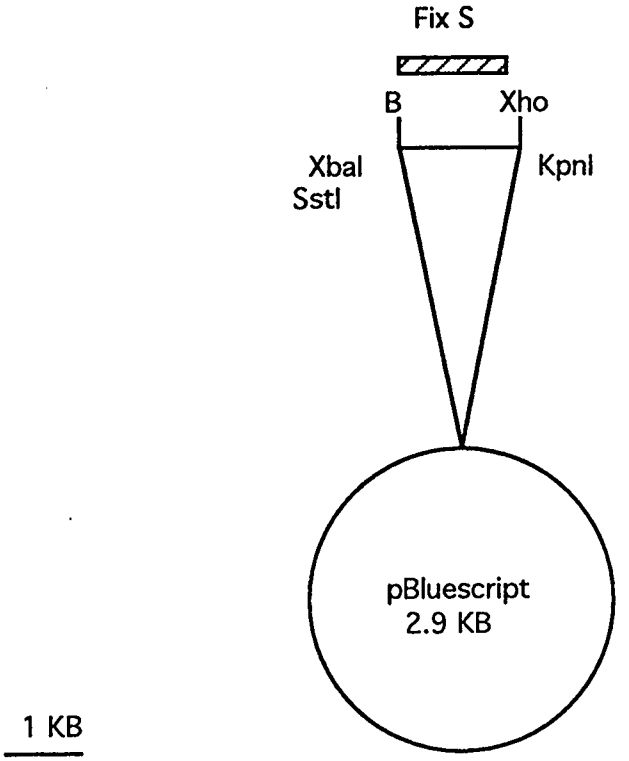
Date: Mar 12/94
Name: pFG93BluSp
Previous titles: 6,10
Source: pSUP401-93Sp
Notes: EcoRI fragment of pSUP401 placed in EcoRI site of pBluescript for insertion into pJQ200SK.



Date: May 5/94
Name: pFG93SpJQ
Previous titles: none
Source: pPFG93BluSp
Notes: XhoI/SstI fragment of pFG93Blu Sp inserted into pJQ200 SK for gene replacement.



Date: Mar 2/95
Name: pFG93-4
Previous titles: #7
Source: Cosmid LB1B2
Notes: Isolated from BamHI fragment containing fix DNA downstream of fix G.



Date: Mar 2/95
Name: pFG93-5
Previous titles: none
Source: cosmid LB1B2
Notes: BamHI/XhoI fragment found to hybridise to fix probes.
Located downstream of fixG and contains remainder of fixS.