# SECOND SITE REVERTANTS OF *fix114* in *R.meliloti*

# GENETIC ANALYSIS OF SECOND SITE REVERTANTS OF *fix114* IN *RHIZOBIUM MELILOTI*

By

IVAN J. ORESNIK, B.Sc

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AUTHOR: Ivan J. Oresnik, B.Sc. (McMaster University)

SUPERVISOR: Professor T. M. Finan

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ABSTRACT:

*R. meliloti* carrying defined deletions that remove *fix114* form Fix$^-$ nodules which are devoid of intracellular bacteria. Occasionally strains which carry these deletions form pink nodules which appear effective in contrast to the normal white ineffective nodules formed by strains carrying *fix114* mutations. Bacteria isolated from these pink nodules retain the original deletion and form effective pink nodules when reinoculated onto alfalfa. It is hypothesized that these isolates carry second site mutations which enable the bacteria to overcome the symbiotic block associated with the *fix114* mutation. In this work, five independent isolates were examined and were shown to carry second site mutations that suppress the symbiotic ineffectiveness completely on alfalfa and incompletely on sweet clover. The five independent second site revertants can be divided into two classes based on genetic data and on their sensitivity to detergents and both classes were localized to the chromosome of the wild type Rm1021. One such second site revertant allele, *sfx-1*, was cloned and localized to a large 18 kb *Bam*HI fragment.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ARA | acetylene reduction activity |
| Ap | ampicillin |
| Bac | bacitracin |
| Cm | chloramphenicol |
| Doc | deoxycholate |
| EPS | exopolysaccharide |
| kb | kilobase pairs of DNA |
| Km | kanamycin |
| LPS | lipopolysaccharide |
| mg | milligram |
| Nm | neomycin |
| Ot | oxytetracycline |
| OD | optical density |
| Rf | rifampicin |
| SE | standard error |
| Sm | streptomycin |
| Sp | spectinomycin |
| Tc | tetracycline |
| X-Gal | 5-bromo-4-chloro-3-indolyl-$\beta$-D-Galactoside |
| X-phos | 5-bromo-4-chloro-3-indolyl phosphate |

# CHAPTER 1

## INTRODUCTION

*Rhizobia* are gram negative rod shaped bacteria that form root nodules with members of the Leguminosae family. The interaction produces root nodules which are capable of reducing di-nitrogen gas to ammonia which is subsequently supplied to the host plant. *Rhizobia* are defined by what plants they are capable of nodulating. For example *Rhizobium leguminosarum* biovar *viciae* nodulates peas, and vetch, while *Rhizobium meliloti* nodulates *Medicago*, *Melilotus*, and *Trigonella* species.

The interaction between *Rhizobia* and the host plant is a complex multistep process that involves both the plant and the microbe (Vincent 1980). The earliest event recognized in *Rhizobium*-Legume symbiosis is the deformation of root hairs. *Rhizobia* attach to root hairs causing a marked root hair curling and the initiation of a nodule meristem in the root cortex. Following root curling the bacteria grow through the plant cells in a plant derived tube called an infection thread. The infection thread continues to grow through the plant cells into the initiated nodule meristem. Once in the nodule meristem, the bacteria are released into the host cytoplasm surrounded by a membrane termed the

"peribacteroid" membrane. In this state the bacteria are termed bacteroids and all nutrients needed for nitrogen fixation are exchanged across this membrane. Mutants that are blocked at different stages have been isolated and these fall into several categories.

Mutants blocked in the earliest stages of nodulation that fail to form any nodules (Nod$^-$) have been described (Long et. al, 1982.). Subsequent analysis of genes involved in nodulation have identified at least twelve nod genes (reviewed in Martinez et al. 1990, Long et al. 1989). The nod genes are divided into "common nod genes" which are found in all Rhizobium species and host specific genes which are restricted to a single Rhizobium species and help define host interaction (Horvath et al. 1987). The nod genes are induced by plant derived flavonoid compounds (Mulligan et al. 1985, Peters et al. 1985, Firmin et al. 1986) that interact with a positive activator, nodD (Mulligan et al. 1985), which in turn appear to bind to a consensus sequence that is upstream of nod promoters termed the "nod box" (Rostas et al. 1986, Hong et al. 1987). The function of the nod genes appears to be the production of a compound that is responsible for root hair curling, infection thread initiation, and nodule initiation (Deballe et al. 1986). Recent work has described the isolation of a factor that is

capable of root hair deformation and nodule initiation that is secreted by R. meliloti (Lerouge et al. 1990).

Mutants have also been isolated that are capable of forming nodule-like structures but either do not form infection threads or are not released from infection threads. Many of these carry mutations associated with the cell surface and will be discussed in a separate section in some detail.

Following infection thread formation, bacteria are released from the infection thread into a nodule cell such that they are surrounded by a plant derived membrane. Mutants that are blocked in symbiosis following release from the infection thread have also been isolated and these fall into several categories and are generally termed nif mutants, fix mutants, and some mutants defective in metabolism.

Mutations that are directly associated with the nitrogenase enzyme (which converts nitrogen gas to ammonia) are termed nif mutants. The nif genes are homologous to previously described nif genes in Klebseilla pneumonia (Buikema et al. 1985 and Buikema et al. 1987). Mutants that fail to fix nitrogen but do not have homology to any known gene directly involved in nitrogen fixation are termed fix mutants. This class is a diverse group of genes that are found on both megaplasmids and the chromosome of R.

meliloti. Some of these appear to be involved in electron transport (Earl et al. 1987), or sensing oxygen concentration (David et al. 1988), while others have no known function as yet (Forrai et al. 1983, Putnokoy et al. 1988).

A third class of genes that are involved in effective symbiosis are those which are associated with carbon and nitrogen metabolism. Examples of these are ntrA (Ronson et al. 1987), dicarboxylate transport (Ronson et al. 1981, Finan et al. 1983, Watson et al. 1988) and some genes which are involved in gluconeogenesis (Finan et al. 1988).

## CELL SURFACE COMPONENTS OF RHIZOBIUM INVOLVED IN SYMBIOSIS

Changes in the cell surface of both plant and bacteria are associated with changes in symbiosis (For examples see Diaz et al. 1989, Glazebrook et al. 1989, Noel et al. 1986). One of the major components of the bacterial cell surface is polysaccharide. This area has been actively investigated since evidence was first presented suggesting microbial polysaccharide interaction with plant lectin (Bohlool and Schmidt 1974). There have been many reports which have either supported or challenged this hypothesis (See Baur 1981 and Dazzo and Truchet 1983 for reviews). Recently pea lectin gene was transformed into white clover and the

resultant roots expressed pea lectin. These plants were nodulated by R. leguminosarum bv. viciae whose host range does not include white clover (Diaz et al. 1989). This is direct evidence that lectins play a role in determining host range.

There are three major polysaccharides that are associated with the cell surface of Rhizobium strains. These are exopolysaccharide (EPS), lipopolysaccharide (LPS), and $\beta$ (1,2) glucan. Genetic evidence suggests that all three play a role in early symbiotic interactions.

## EXOPOLYSACCHARIDES

Exopolysaccharides are defined as polysaccharides that are not attached to the cell surface. These are usually responsible for the slimy colony morphology exhibited by Rhizobium. The best characterized system for exopolysaccharide production is in R. meliloti. Two types of exopolysaccharide, EPS I and EPS II, have been identified. Defined mutants of R. meliloti in exopolysaccharide synthesis (EPS I) were described by Finan et al. (1985), Leigh et al. (1985), and by Muller et al. (1988). Genetic analysis of these mutants revealed that many mutations in EPS I synthesis resulted in an ineffective ($Fix^-$) phenotype which produced nodules devoid of bacteria when inoculated onto alfalfa.

Another class of EPS mutant, exoH, produces an EPS that differs from wild type by a single succinylation, also forms ineffective nodules (Leigh et al. 1987). This suggests that not only is the production of EPS important, but that EPS must have a specific structure.

EPS I mutations are found on a second megaplasmid in R. meliloti (Finan et al. 1986, Hynes et al. 1986) and two loci, exoC and exoD, are chromosomally-located (Finan et al. 1986). The exopolysaccharide genes found on the second megaplasmid of Rm1021 are part of a large cluster of genes spanning 20 kb and consisting of 12 complementation groups (Long et al. 1988a). Of these, three, exoB, exoF, and exoP, are membrane- associated proteins that give active TnphoA fusions (Long et al. 1988b). exoB mutants (Finan et al. 1985, Leigh et al. 1985) are the most pleiotropic mutants, being insensitive to a number of phage and having an altered lipopolysaccharide (Finan et al. 1985, Leigh et al. 1988). The exoC mutants also have pleiotropic phenotypes and do not produce any $\beta$ (1,2) glucan (Leigh et al. 1988).

Regulators of EPS genes have also been described in R. meliloti and R. leguminosarum bv. phaseoli (Doherty et al. 1988, Borthakur et al. 1985). In R. meliloti, negative regulators were isolated by Tn5 mutagenesis and are chromosomally-located (Doherty et al. 1988). In R. leguminosarum bv. phaseoli, a gene, psi (polysaccharide

inhibition), was isolated that is required for nitrogen fixation and is thought to regulate EPS synthesis in the bacteroid state (Borthakur et al. 1985). The psi gene is thought to interact with another gene, psr (polysaccharide restoration), which represses psi in the free living state bacteria (Borthakur et al. 1987). Wild type strains that carry psr on a multicopy plasmid are found to be ineffective (Borthakur et al. 1987). The relationship between the regulatory genes in R. leguminosarum bv. phaseoli and EPS mutants in R. meliloti is unclear at present.

Although exopolysaccharride (EPS I) in R. meliloti SU47 is necessary for effective nodulation, a second exopolysaccharide (EPS II) has also been described (Glazebrook et al. 1989, Zahn et al. 1989). Glazebrook and Walker (1989) and Zahn et al. (1989) describe mutations which result in an increased expression of a second saccharide (EPS II) in R. meliloti. The mutations, however, mapped to different chromosomal loci. The mutation described by Glazebrook and Walker (1989), expR101, mapped counter-clockwise from trp-33 whereas the mutation described by Zahn et al. (1989), mucR12, mapped counter clockwise from pyr49 in Rm1021. In both cases, the second saccharide (EPS II) which is expressed maps to the second megaplasmid of R. meliloti described by Finan et al. (1986). The structure of EPS II appears to be the same in both reports. However,

Zahn et al. (1989) report that the second saccharide (EPS II) functionally replaces the first saccharide (EPS I) on both alfalfa and sweet clover, but Glazebrook and Walker (1989) report suppression as an alfalfa-specific event. It is not clear whether these differences are real or are due to the experimental methods used. The evidence, however, still suggests that exopolysaccharides may play a role in determining the host range of Rhizobium meliloti.

Further evidence that EPS may help determine host range comes from a mutation which was described in R. leguminosarum bv.phaseoli. A Tn5 mutant which is EPS$^-$ in R. leguminosarum bv. phaseoli was effective when inoculated onto beans but when this mutation was moved into a near-isogenic strain of R. leguminosarum bv. viciae, the strain was completely ineffective when inoculated onto peas (Borthakur et al. 1986). A similar result was also reported by Diebold et al. (1989). Although the role for EPS in symbiosis is unknown, other strains of Rhizobium that are defective in EPS also appear to show defects in symbiosis (Chakroverty et al. 1982, Chen et al. 1985, Napoli and Albersheim 1980). The only exception is Rhizobium leguminosarum bv. phaseoli where EPS appears not to be necessary (Diebold et al. 1989, Borthakur et al. 1986). A possible explanation suggested that EPS may not be necessary for determinant nodule symbiosis (Diebold et al. 1989).

This is supported by another report that shows Bradyrhizobium japonicum EPS$^-$ mutants are also effective (Law et al. 1982).

## LIPOPOLYSACCHARIDES OF RHIZOBIUM

Lipopolysaccharides (LPS) of Rhizobium, E. coli and Salmonella are similar in structure (Carlson 1984, Carlson et al. 1983, Zevenhuizen et al. 1980). Each contains a lipid A moiety, 2-keto,3-deoxyoctonic acid (KDO), which links lipid A with a core polysaccharide, and an O antigen polysaccharide that is attached to the core polysaccharide (Figure 1). LPS among Rhizobium appears quite heterogeneous with LPS varying between strains as much as between species (Carlson 1984, Zevenhuizen 1980).

A component of the outer cell membrane LPS is thought of as a receptor or determinant for nodulation specificity by many people. Early evidence suggested that LPS from R. leguminosarum bv. viciae specifically bound pea lectin whereas LPS from other Rhizobium species did not bind pea lectin (Kato et al. 1979). As well a similar result was reported for R. meliloti and alfalfa lectin (Kanberger et al. 1970). Hraback et al. (1981) reported that R. trifolii had multiple forms of LPS that are dependent upon the growth phase of the bacterial culture, one of which appeared to be a receptor for clover lectin trifollin A. Although binding

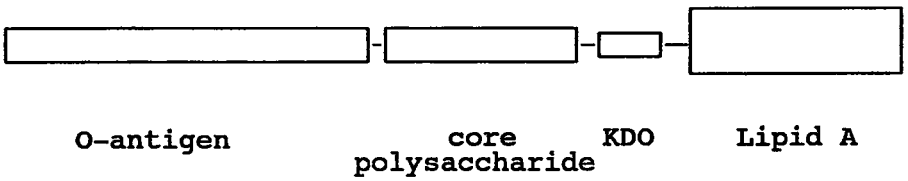



Figure 1. Schematic representation of LPS o-antigen/core polysaccharide are linked saccharides linked to 3-deoxy-D mannooctulosonic acid (KDO) . KDO is linked to a Lipid A residue which consists of glucosamine residues linked to fatty acids which are anchored in the outer bacterial membrane.

studies suggested LPS interaction with the host plant, evidence linking LPS with a direct role in symbiosis could not be firmly established.

Maier and Brill (1976) reported symbiotically deficient mutants that were isolated following a chemical mutagenesis and screening of 2,500 survivors. Analysis of two such mutants showed that they appeared to have an altered O antigen indicating that LPS may have a role in symbiosis (Maier and Brill 1978). Because these mutants were generated by chemical mutagenesis these results should be viewed with caution since the nature of the mutation has not been defined.

In R. leguminosarum bv. phaseoli the first well defined Tn5 mutants defective in LPS were isolated (Noel et al. 1986). These mutants are ineffective when inoculated onto bean plants and the symbiotic defect appeared to be during infection thread development (Noel et al. 1986). Biochemical characterization of these mutants showed that they are missing the O antigen (Carlson et al. 1987).

Brewin et al. (1986) isolated monoclonal antibodies to LPS of R. leguminosarum bv. viciae both from free-living cells and bacteroids and in a subsequent report presented evidence for LPS interacting with the peribacteroid membrane (Bradley et al. 1986). Further work has shown that a monoclonal antibody that recognizes LPS in R. leguminosarum

bv. viciae bacteroids is differentially expressed within the infection thread and the infected nodule cells but not expressed in the free living state (Vanden Bosch et al. 1989). This antigen was also shown to be posotively regulated by low pH or low oxygen concentrations (Kannenberg et al. 1989). Mutants that constitutively express this antigen however are still effective when inoculated onto peas (Wood et al. 1989). The corollary to this work is that LPS appears to change from the free living to the bacteroid state (Vanden Bosch et al. 1989).

Mutants in R. leguminosarum bv. viciae that have an altered LPS have also been isolated (Prieffer 1989, deMaagd et al. 1989). These mutants are ineffective symbiotically and are blocked in either infection thread development or shortly after release into the plant cell. It is not known how the LPS produced by these mutants compares with the LPS antigens studied by Wood et al. (1989).

Mutants which have altered LPS have also been described in R. meliloti (Clover et al. 1989). These mutants were isolated as being resistant to phage or sensitive to the detergent deoxycholate which is diagnostic for LPS alterations in enteric bacteria (Sanderson et al. 1974). These mutants are effective in a wild type background when inoculated onto alfalfa but the biochemical defect in the LPS was not determined (Clover et al. 1989). In another

report, however, an alteration in LPS suppresses an exopolysaccharide deficiency in another R. meliloti strain (Williams et al. 1990) suggesting that LPS does play a role in the symbiotic effectiveness of R. meliloti.

It appears that LPS does play a role in the symbiosis of Rhizobium. The precise mechanism, however, may vary depending upon the species and the host plant that is being studied.

## CYCLIC β (1,2) GLUCANS IN RHIZOBIUM

Cyclic β (1,2) glucans have been described as being associated with Rhizobium (Zevenhuizen et al. 1979, York et al. 1980). These molecules have been localized to the periplasm of Rhizobium trifolii (Abe et al. 1982) and to the periplasm of the closely related bacteria Agrobacterium tumefaciens (Miller et al. 1986). Cyclic β (1,2) glucan consists of 17-21 glucose units linked together by β 1,2 linkages to form a large circular molecule (Zevenhuizen et al. 1979, York et al. 1980). Cyclic β (1,2) glucan has been suggested to be analogous to membrane-derived oligosaccharides (mdo) of E. coli because they are similar in structure and their synthesis is induced by low osmolarity (Miller et al. 1986).

Genes for nodule development (ndv) were isolated as being homologous to A. tumefaciens chromosomal virulence (chv) chvA and chvB genes (Dylan et al. 1986). The ndv genes can functionally replace chvA and chvB and that R. meliloti mutated in either ndvA or ndvB formed ineffective white nodules that were devoid of bacteria (Dylan et al. 1986). In A. tumefaciens, mutations in chvB are pleiotropic and affect attachment to plant cells, motility, and virulence (Douglas et al. 1982). This was also shown to be true for R. meliloti ndv mutants (Dylan et al. 1986). In A. tumefaciens, evidence for chvB involvement in cyclic β (1,2) glucan synthesis was reported (Puvanesarajah et al. 1985). By analogy ndv was shown also to be involved in cyclic β (1,2) glucan synthesis (Stanfield et al. 1988, Ielpi et al. 1990).

Sequence analysis of ndvA indicates that this gene encodes for a 67.1 kilodalton protein that is homologous to haemolysin B (HylB) which is involved in the export of haemolysin in E. coli (Stanfield et al. 1988). This suggests that ndvA may encode a protein that is involved in the export of β 1,2 glucan across the membrane of R. meliloti. A similar conclusion was also made for chvA in A. tumefaciens (O'Connel et al. 1988).

The ndvB gene is believed to code for a 235 kilodalton protein that is also involved in the synthesis of β (1,2)

glucan (Geremia et al. 1987, Ielpi et al. 1990). Zorreguieta et al. (1986) showed that β (1,2) glucan synthesis involves a large 235 kilodalton membrane associated protein. A mutant generated by heat treatment in R. meliloti was missing a 235 kilodalton protein and is defective in β (1,2) glucan synthesis (Geremia et al. 1987). These mutants, however, were not shown to be the same as the ndv mutants described by Dylan et al. (1986) (Geremia et al. 1987). Sequencing and biochemical characterization of ndvB demonstrated that this locus encodes a large 319 kilodalton membrane-associated protein that is involved in the synthesis of β (1,2) glucan (Ielpi et al. 1990).

The biological role of β (1,2) glucan in A. tumefaciens appears to be an osmotic adaptation by analogy to E. coli (Miller et al. 1986). Exogenously added β (1,2) glucan to Rhizobium cultures appears to enhances nodulation (Abe et al. 1982, Dylan et al. 1990). It does not, however, reverse either the ndvA or ndvB symbiotic phenotype (Dylan et al. 1990).

R. meliloti ndvA and ndvB mutants have an osmotic phenotype that includes reduced growth rate, reduced motility, phage sensitivity, and antibiotic sensitivity at low osmolarity (Dylan et al. 1986, Dylan et al. 1990). All of these phenotypes can be reversed by an increase in the osmolarity of the media (Dylan et al. 1990). The

conclusions of this work were that β (1,2) glucans are synthesised in response to low osmolarity, can be found in the periplasm, and are essential in the osmoadaption of R. meliloti (Dylan et. al. 1990).

Further work with ndvA and ndvB reported the isolation of pseudorevertants of ndvA and ndvB (Dylan et al. 1990b). These pseudorevertants fell into two classes: symbiotic pseudorevertants, and motile revertants. Neither of these classes regained the ability to synthesize β (1,2) glucan. The motile class had lost their osmotic phenotype although they were still ineffective symbiotically whereas the symbiotic pseudorevertants were effective when reinoculated on alfalfa, but still had an osmotic phenotype (Dylan et al. 1990b). This evidence suggests that β (1,2) glucan is not required directly for effective symbiosis (Dylan et al. 1990b).

## SUPPRESSION OF SYMBIOTIC PHENOTYPES IN R. MELILOTI

Suppression of ineffective symbiosis which are due to cell surface alterations have been recently described in the literature (Glazebrook and Walker 1989, Zahn et al. 1989, Dylan et al. 1990b, Williams et al. 1990). A second cryptic exopolysaccharide that can functionally replace the first exopolysaccharide on alfalfa (Glazebrook and Walker 1989) or on alfalfa and sweet clover (Zahn et al. 1989) has been

described. Dylan et al. (1990b) described the isolation of symbiotic pseudorevertants which were able to suppress the ndv mutants on alfalfa. The mechanism of suppression is not a simple restoration of cyclic β (1,2) glucan production and does not alleviate the osmotic phenotype associated with ndv mutants (Dylan et al. 1990b).

AK631 was originally isolated as a compact colony wild type of R. meliloti strain Rm41 (Forrai et al. 1983). Recently it has been reported that AK631 carries a mutation that blocks EPS synthesis (Putnoky et al. 1988). Mutations in exopolysaccharide synthesis, however, are generally ineffective in R. meliloti (Leigh et al. 1985). Further analysis of this anomaly showed that AK631 did not produce EPS I or EPS II, carried an exoB type mutation, and the plant associated phenotype was suppressed by another gene, lpsZ$^{+}$. Introduction of this gene resulted in the production of an altered lipopolysaccharide which functionally replaces exopolysaccharide in R. meliloti in symbiosis (Williams et al. 1990, Putnoky et al. 1990). These examples are precedent for the idea that alterations in the cell surface of Rhizobium can either be replaced functionally or masked by secondary mutations that allow effective symbiosis.

## FIX114

Genetic analysis of the second megaplasmid, pRmeSU47b, of R. meliloti Rm1021 by the use of large defined deletions resulted in the identification of two overlapping deletions, ΔF114 and Δ5408, both of which resulted in the formation of Fix⁻ nodules. Subsequent analysis defined the locus fix114 which is absent in both ΔF114 and Δ5408 (Figure 2). The wild type region was isolated from a cosmid bank of wild type DNA (Freidman et al. 1985) and the fix114 locus was delimited to 5 kb by Tn5 mutagenesis and sub-cloning (Figure 3). TnphoA mutagenesis of the fix114 region indicates that this region codes for protein(s) that have periplasmic domains (Figure 3).

Mutations or deletions of the fix114 locus result in several phenotypes. Rhizobium meliloti carrying mutations at this locus form white, ineffective nodules that are devoid of bacteria (W. Newcomb personal communication). Strains carrying an 8 kb deletion removing the fix114 locus or single insertions in this locus give a mucoid phenotype on low osmolarity media. Rhizobium meliloti carrying either ΔF114 or Δ5408 also have an ineffective symbiotic phenotype and reduced motility but do not appear to have a phage phenotype which differs from the wild type.

At the time this work was started it was observed that plants inoculated with strains carrying ΔF114 or Δ5408

Figure 2. Map of pRmeSU47b showing positions of ΔF114, Δ5408 and fix-114.

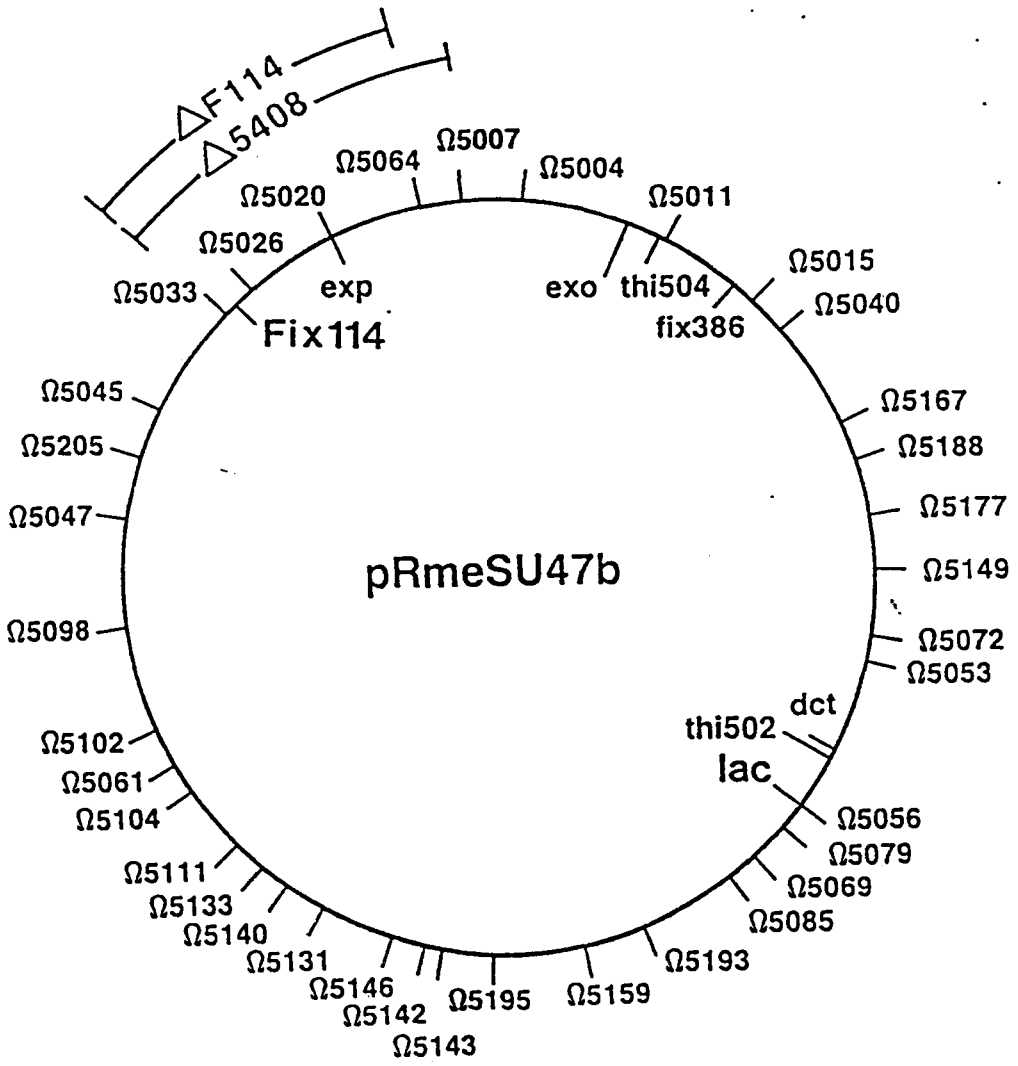
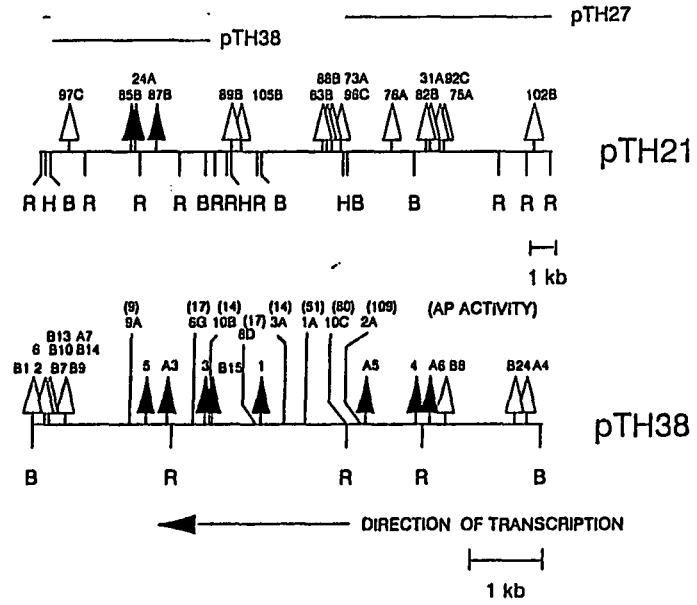

ΔF114
Δ5408
Ω5064
Ω5007
Ω5004
Ω5020
Ω5011
Ω5026
Ω5015
Ω5033
exp
exo
thi504
Ω5040
Fix114
fix386
Ω5045
Ω5167
Ω5205
Ω5188
Ω5047
Ω5177
pRmeSU47b
Ω5149
Ω5098
Ω5072
Ω5053
dct
thi502
Ω5102
lac
Ω5061
Ω5056
Ω5104
Ω5079
Ω5111
Ω5069
Ω5133
Ω5085
Ω5140
Ω5193
Ω5131
Ω5146
Ω5159
Ω5195
Ω5142
Ω5143

Figure 3. Restriction map of cosmid pTH21 and subclone pTH38 carrying *fix-114* region showing positions of Tn*5* and active Tn*phoA* insertions (From T. Charles Ph.D. thesis McMaster University).


pTH27
pTH38
97C
24A
85B 87B
89B 105B
88B 73A
63B 96C
76A
31A92C
82B 75A
102B
pTH21
R H B R R R BRRHR B HB B R R R
1 kb
(9)
9A
(17) (14)
6G 10B
(17)
8D
(14)
3A
(51) (80) (109)
1A 10C 2A
(AP ACTIVITY)
B13 A7
6 B10 B14
B1 2 B7 B9
5 A3
3
B15
1
A5
4 A6 B8
B24 A4
pTH38
B R R R B
DIRECTION OF TRANSCRIPTION
1 kb
Fix+ Tn5 insertion
Fix− Tn5 insertion
TnphoA insertion

occasionally formed pink $Fix^+$ nodules. The $Fix^+$ phenotype was shown to result from second site mutations which suppressed the $Fix^-$ phenotype of strains carrying ΔF114 and Δ5408. The goal of this thesis was to characterize these second site mutations in an effort to establish the role of fix114 in symbiosis. These second site mutations were divided into two distinct classes based on phenotype and mapping data. One such suppressor allele, sfx-1 has been cloned and it has been shown to reverse the symbiotic phenotype of ΔF114 and Δ5408 and the osmotic phenotype associated with the fix114 locus.

# CHAPTER 2

## Bacterial strains, phage, and plasmids

All bacterial strains and phage that were used are found in Table 1. Relevant genotypes, phenotypes and references are shown. Plasmids that were used as cloning vectors, or were isolated in this work, as well as their descriptions are also listed in Table 1.

All Rhizobium meliloti strains were grown at 30°C. Escherichia coli cultures were grown at 37°C. Occasionally overnight broth cultures were grown at 30°C and subcultured before use.

## MEDIA

All media used for the growth of bacterial cultures was sterilized by autoclaving at 15 pounds/square inch, at least 121°C, for at least 15 minutes.

Complex media for R. meliloti and E. coli was LB (Miller 1972) containing 10 g tryptone, 5 g Yeast extract, and 5 g NaCl per litre of water. For broth cultures NaOH was added to a concentration of 4 mM before autoclaving. For LB agar, NaOH was added to a concentration of 1 mM and

TABLE 1. Bacterial strains, plasmids, phage, and transposons

| Strain,plasmid, or transposon | Relevant Characteristics | Source,reference,or construction |
|---|---|---|
| *Rhizobium meliloti* | | |
| Rm1021 | SU47 *str-21* | Meade et al. 1982 |
| Rm5000 | SU47 *rif-5* | Finan et al. |
| *Transposon insertion banks* | | |
| Bank NM1 | ca. 6000 Tn*5* insertions in Rm1021 background | T. Charles |
| Bank GS2 | ca. 2000 Tn*5*-233 insertions in Rm5000 background | T. Charles |
| Bank OT3 | ca. 1000 Tn*5*-132 insertions in Rm1021 background | A. Bottacin |
| *Rm1021 derivatives*[a] | | |
| DDX19 | *trp-514*::Tn*5* | T. Finan |
| Rm5320 | Rm1021 Ω30::Tn*5*-11 | T. Finan |
| Rm5348 | Rm1021 Ω5025::Tn*5* | T. Charles |
| Rm5439 | *pck-1*::Tn*V* | Finan et al. 1988 |
| Rm5408 | ΔΩ5033-5077::Tn*5*-233 | Φ(Rm5356)->Rm5393,$Gm^r$-$Sp^r$ $Nm^s$ |
| Rm6661 | SU47,*his-39, trp-33, leu-53*$^{\pm}$ Ω601::Tn*5*-*mob*(-) | S. Klein |
| Rm6662 | SU47,*his-39, trp-33, leu-53*$^{\pm}$ Ω602::Tn*5*-*mob*(+) | S. Klein |
| Rm6692 | SU47,*his-39, leu-53, trp-33*$^{\pm}$ Ω611::Tn*5*-*mob*(+) | S. Klein |

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| Rm6693 | SU47,*his-39, leu-53, trp-33*$^{\pm}$ Ω612::Tn*5-mob*(-) | S. Klein |
| Rm6695 | SU47,*his-39, trp-33, pyr-49*$^{\pm}$ Ω614::Tn*5-mob*(+) | S. Klein |
| Rm6696 | SU47,*his-39, trp-33, pyr-49*$^{\pm}$ Ω615::Tn*5-mob*(-) | S. Klein |
| Rm6865 | SU47, *his-39, trp-33, pyr-49, cys-11*$^{\pm}$ Ω637::Tn*5-mob* (+) | S. Klein |
| Rm7013 | *exoB13*::Tn*5* | Finan et al. 1985. |
| Rm7031 | *exoA31*::Tn*5* | Leigh et al. 1985. |
| Rm7055 | *exoF55*::Tn*5* | Leigh et al. 1985. |
| RmF114 | ΔΩ5033-5064::Tn*5*-233 | T. Charles |
| RmF123 | 1021 Ω5047::Tn*5*-11 (-) | T. Charles |
| RmF124 | 1021 Ω5047::Tn*5*-11 | T. Charles |
| RmF222 | =Rm8002 *phoA* | Long *et. al 1988b* |
| RmF263 | ΔΩ5033-5077::Tn*5*-233, *sfx-1* | Fix$^{+}$ nodule isolate from Rm5408, T. Charles |
| RmF338 | ΔΩ5033-5077::Tn*5*-233, Ω5117::Tn*5*, *sfx-1* | This work (see Fig 4) |
| RmF339 | ΔΩ5033-5077::Tn*5*-233, Ω5118::Tn*5*, *sfx-1* | This work (see Fig 4) |
| RmF346 | ΔΩ5033-5064::Tn*5*-233, *sfx-2* | Fix$^{+}$ nodule isolate from RmF114, T. Charles |
| RmF378 | Ω5117::Tn*5* | Φ(RmF338)->Rm1021, Nm$^{r}$ Gm$^{s}$-Sp$^{s}$ |
| RmF379 | Ω5118::Tn*5*, *sfx-1* | Φ(RmF339)->Rm1021, Nm$^{r}$ Gm$^{s}$-Sp$^{s}$ |

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| RmF418 | Ω5117::Tn*5*, Ω5047::Tn*5*-11(+) | Φ(RmF378)->RmF123, $Nm^r$ $Gm^r$-$Sp^r$ |
| RmF419 | Ω5117::Tn*5*, Ω5047::Tn*5*-11(-) | Φ(RmF378)->RmF124, $Nm^r$ $Gm^r$-$Sp^r$ |
| RmF420 | Ω5117::Tn*5*, Ω30::Tn*5*-11(+) | Φ(RmF378)->Rm5320, $Nm^r$ $Gm^r$-$Sp^r$ |
| RmF421 | Ω5118::Tn*5*, Ω5047::Tn*5*-11(+) | Φ(RmF379)->RmF123, $Nm^r$ $Gm^r$-$Sp^r$ |
| RmF422 | Ω5118::Tn*5*, Ω5047::Tn*5*-11(-) | Φ(RmF379)->RmF124, $Nm^r$ $Gm^r$-$Sp^r$ |
| RmF423 | Ω5118::Tn*5*, Ω30::Tn*5*-11(+) | Φ(RmF379)->Rm5320, $Nm^r$ $Gm^r$-$Sp^r$ |
| RmF424 | ΔΩ5033-5077::Tn*5*-233, Ω5119::Tn*5*-132 | Φ(BankOT3)->RmF339, $Gm^r$-$Sp^r$ $Ot^r$ $Nm^s$ $Fix^-$ |
| RmF425 | ΔΩ5033-5077::Tn*5*-233, Ω5120::Tn*5*-132, *sfx-1* | Φ(BankOT3)->RmF339, $Gm^r$-$Sp^r$ $Ot^r$ $Nm^s$ $Fix^+$ |
| RmF426 | ΔΩ5033-5077::Tn*5*-233, Ω5121::Tn*5*-132, *sfx-1* | Φ(BankOT3)->RmF339, $Gm^r$-$Sp^r$ Otr $Nm^s$ $Fix^+$ |
| RmF427 | ΔΩ5033-5077::Tn*5*-233, Ω5122::Tn*5*-132 | Φ(BankOT3)->RmF339, $Gm^r$-$Sp^r$ $Ot^r$ $Nm^s$ $Fix^-$ |
| RmF428 | ΔΩ5033-5077::Tn*5*-233, Ω5123::Tn*5*-132, *sfx-1* | Φ(BankOT3)->RmF339, $Gm^r$-$Sp^r$ $Ot^r$ $Nm^s$ $Fix^+$ |
| RmF429 | ΔΩ5033-5077::Tn*5*-233, Ω5124::Tn*5*-132, *sfx-1* | Φ(BankOT3)->RmF339, $Gm^r$-$Sp^r$ Otr $Nm^s$ $Fix^+$ |
| RmF430 | ΔΩ5033-5077::Tn*5*-233, Ω5125::Tn*5*-132, *sfx-1* | Φ(BankOT3)->RmF338, $Gm^r$-$Sp^r$ $Ot^r$ $Nm^s$ $Fix^+$ |
| RmF431 | ΔΩ5033-5077::Tn5233, Ω5126::Tn*5*-132, *sfx-1* | Φ(BankOT3)->RmF338, $Gm^r$-$Sp^r$ $Ot^r$ $Nm^s$ $Fix^+$ |
| RmF919 | *fix114-1*::Tn*5* | T. Charles |
| RmF921 | *fix114-3*::Tn*5* | T. Charles |

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| RmF922 | *fix114-5*::Tn*5* | T. Charles |
| RmF993 | Ω5117::Tn*5*-233 | Tn*5*-233 replacement of Ω5117::Tn*5* in RmF378 |
| RmF994 | Ω5118::Tn*5*-233, *sfx-1* | Tn*5*-233 replacement of Ω5118 in RmF379 |
| RmG120 | Ω5117::Tn*5*-233, Ω601::Tn*5*-*mob*(-) | Φ(Rm6661)->RmF993, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG121 | Ω5117::Tn*5*-233, Ω602::Tn*5*-*mob*(+) | Φ(Rm6662)->RmF993, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG122 | Ω5117::Tn*5*-233, Ω611::Tn*5*-*mob*(+) | Φ(Rm6692)->RmF993, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG123 | Ω5117::Tn*5*-233, Ω612::Tn*5*-*mob*(-) | Φ(Rm6693)->RmF993, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG124 | Ω5117::Tn*5*-233, Ω614::Tn*5*-*mob*(+) | Φ(Rm6695)->RmF993, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG125 | Ω5117::Tn*5*-233, Ω615::Tn*5*-*mob*(-) | Φ(Rm6696)->RmF993, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG126 | Ω5117::Tn*5*-233, Ω637::Tn*5*-*mob*(+) | Φ(Rm6865)->RmF993, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG127 | Ω5118::Tn*5*-233, Ω601::Tn*5*-*mob*(-) | Φ(Rm6661)->RmF994, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG128 | Ω5118::Tn*5*-233, Ω602::Tn*5*-*mob*(+) | Φ(RM6662)->RmF994, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG129 | Ω5118::Tn*5*-233, Ω611::Tn*5*-*mob*(+) | Φ(Rm6692)->RmF994, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG130 | Ω5118::Tn*5*-233, Ω612::Tn*5*-*mob*(-) | Φ(Rm6693)->RmF994, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG131 | Ω5118::Tn*5*-233, Ω614::Tn*5*-*mob*(+) | Φ(Rm6695)->RmF994, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG132 | Ω5118::Tn*5*-233, Ω615::Tn*5*-*mob*(-) | ΦRm(6696)->RmF994, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG133 | Ω5118::Tn*5*-233, Ω637::Tn*5*-*mob*(+) | Φ(Rm6865)->RmF994, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG134 | Ω5117::Tn*5*, Ω5118::Tn*5*-233 | Φ(RmF378)->RmF994, $Nm^r$, $Gm^r$-$Sp^r$ |

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| RmG135 | Ω5117::Tn*5*-233, Ω5118::Tn*5* | Φ(RmF379)->RmF993, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG156 | *fix114-1*::Tn*5*, *sfx-1* | T. Charles |
| RmG158 | *fix114-4*::Tn*5*, *sfx-1* | T. Charles |
| RmG159 | *fix114*-5::Tn*5*, *sfx-1* | T. Charles |
| RmG164 | Ω5119::Tn*5*-132 | Φ(RmF424)->Rm1021, $Ot^r$ |
| RmG165 | Ω5120::Tn*5*-132 | Φ(RmF425)->Rm1021, $Ot^r$ |
| RmG166 | Ω5121::Tn*5*-132 | Φ(RmF426)->Rm1021, $Ot^r$ |
| RmG167 | Ω5122::Tn*5*-132 | Φ(RmF427)->Rm1021, $Ot^r$ |
| RmG169 | Ω5124::Tn*5*-132 | Φ(RmF429)->Rm1021, $Ot^r$ |
| RmG170 | Ω5125::Tn*5*-132 | Φ(RmF430)->Rm1021, $Ot^r$ |
| RmG171 | Ω5126::Tn*5*-132 | Φ(RmF431)->Rm1021, $Ot^r$ |
| RmG199 | Rm1021 Ω5121::Tn*V* | Tn*V* replacement of Ω5121::Tn*5*-132 in RmG166 |
| RmG200 | Rm1021 Ω5123::Tn*V* | Tn*V* replacement of Ω5123::Tn*5*-132 in RmG168 |
| RmG201 | Rm1021 Ω5124::Tn*V* | Tn*V* replacement of Ω5124::Tn*5*-132 in RmG169 |
| RmG202 | Rm1021 Ω5126::Tn*V* | Tn*V* replacement of Ω5126::Tn*5*-132 in RmG170 |
| RmG203 | ΔΩ5033-5077::Tn*5*-233, *sfx-4* | $Fix^+$ nodule isolate from Rm5408 |
| RmG204 | ΔΩ5033-5064::Tn*5*-233, *sfx-5* | $Fix^+$ nodule isolate from RmF114 |

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| RmG205 | Ω5118::Tn*5*-233, *trp-514*::Tn*5* | Φ(DDX19)->RmF994, $Nm^r$, $Gm^r$-$Sp^r$ |
| RmG256 | Ω5117::Tn*5*, Ω5122::Tn*5*-132 | Φ(RmF378)->RmG167, $Nm^r$, $Ot^r$ independent isolate |
| RmG257 | Ω5117::Tn*5*, Ω5122::Tn*5*-132 | Φ(RmF378)->RmG167, $Nm^r$, $Ot^r$ independent isolate |
| RmG258 | Ω5117::Tn*5*, Ω5122::Tn*5*-132 | Φ(RmF378)->RmG167, $Nm^r$, $Ot^r$ independent isolate |
| RmG259 | Ω5117::Tn*5*, Ω5122::Tn*5*-132 | Φ(RmF378)->RmG167, $Nm^r$, $Ot^r$ independent isolate |
| RmG260 | Ω5122::Tn*5*-235 | Indepeendent replacement of Ω5122::Tn*5*-132 in RmG167 |
| RmG261 | Ω5122::Tn*5*-235 | Independent replacement of Ω5122::Tn*5*-132 in RmG167 |
| RmG324 | ΔΩ5033-5077::Tn*5*-233, Ω5117::Tn*5*, Ω5122::Tn*5*-132 | Φ(RmG256)->RmF263, $Nm^r$ $Ot^r$, $Gm^r$-$Sp^r$ $Fix^-$ |
| RmG325 | ΔΩ5033-5077::Tn*5*-233, Ω5117::Tn*5*, Ω5122::Tn*5*-132 | Φ(RmG256)->RmF263, $Fix^-$ $Nm^r$, $Ot^r$, $Gm^r$-$Sp^r$ |
| RmG328 | ΔΩ5033-5064::Tn*5*-233, Ω5117::Tn*5*, Ω5122::Tn*5*-132, *sfx-2* | Φ(RmG256)->RmF346, $Nm^r$, $Ot^r$, $Gm^r$-$Sp^r$ $Fix^+$ |
| RmG329 | ΔΩ5033-5064::Tn*5*-233, Ω5117::Tn*5*, Ω5122::Tn*5*-132, *sfx-2* | Φ(RmG256)->RmF346, $Nm^r$, $Ot^r$, $Gm^r$-$Sp^r$ $Fix^+$ |
| RmG330 | ΔΩ5033-5077::Tn*5*-233, Ω5117::Tn*5*, Ω5122::Tn*5*-132 | Φ(RmG256)->RmG203, $Nm^r$, $Ot^r$, $Gm^r$-$Sp^r$ $Fix^-$ |

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| RmG331 | ΔΩ5033-5077::Tn*5*-233, Ω5117::Tn*5*, Ω5122::Tn*5*-132 | Φ(RmG256)->RmG203, $Nm^r$, $Ot^r$, $Gm^r$-$Sp^r$ $Fix^-$ |
| RmG332 | ΔΩ5033-5064::Tn*5*-233, Ω5117::Tn*5*, Ω5122::Tn*5*-132 | Φ(RmG256)->RmG204, $Nm^r$, $Ot^r$, $Gm^r$-$Sp^r$ $Fix^-$ |
| RmG333 | ΔΩ5033-5064::Tn*5*-233, Ω5117::Tn*5*, Ω5122::Tn*5*-132 | Φ(RmG256)->RmG204, $Nm^r$, $Ot^r$, $Gm^r$-$Sp^r$ $Fix^-$ |
| RmG334 | Ω5226::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG335 | Ω5227::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG336 | Ω5228::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG337 | Ω5229::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG338 | Ω5230::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG339 | Ω5231::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG340 | *phe-232*::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG341 | Ω5233::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG342 | Ω5234::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG343 | Ω5235::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG344 | Ω5236::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG345 | Ω5237::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG346 | Ω5238::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG347 | Ω5239::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |

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| RmG348 | Ω5240::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG349 | Ω5241::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG350 | Ω5242::Tn*5*-233 | Φ(BankGS2)->RmF378, $Gm^r$-$Sp^r$ $Nm^s$ |
| RmG351 | Ω5343::Tn*5*-233 | Φ(BankGS2)->RmG167, $Gm^r$-$Sp^r$ $Ot^s$ |
| RmG352 | Ω5244::Tn*5*-233 | Φ(BankGS2)->RmG167, $Gm^r$-$Sp^r$ $Ot^s$ |
| RmG353 | Ω5245::Tn*5*-233 | Φ(BankGS2)->RmG167, $Gm^r$-$Sp^r$ $Ot^s$ |
| RmG354 | Ω5246::Tn*5*-233 | Φ(BankGS2)->RmG167, $Gm^r$-$Sp^r$ $Ot^s$ |
| RmG355 | Ω5247::Tn*5*-233 | Φ(BankGS2)->RmG167, $Gm^r$-$Sp^r$ $Ot^s$ |
| RmG356 | Ω5248::Tn*5*-233 | Φ(BankGS2)->RmG167, $Gm^r$-$Sp^r$ $Ot^s$ |
| RmG357 | Ω5249::Tn*5*-233 | Φ(BankGS2)->RmG167, $Gm^r$-$Sp^r$ $Ot^s$ |
| RmG358 | Ω5250::Tn*5*-233 | Φ(BankGS2)->RmG167, $Gm^r$-$Sp^r$ $Ot^s$ |
| RmG359 | Ω5251::Tn*5*-233 | Φ(BankGS2)->RmG167, $Gm^r$-$Sp^r$ $Ot^s$ |
| RmG360 | Ω5252::Tn*5*-233 | Φ(BankGS2)->RmG167, $Gm^r$-$Sp^r$ $Ot^s$ |
| RmG360A | Ω5253::Tn*5*-233 | Φ(BankGS2)->RmG167, $Gm^r$-$Sp^r$ $Ot^s$ |
| RmG425 | ΔΩ5033-5064::Tn*5*-233, *sfx-3* | $Fix^+$ nodule isolate from RmF114 |
| RmG439 | pTH22 Δ*Hin*dIII::Nm (13 kb) | T. Charles |
| RmG490 | *fix114-1.7*Ω $Sp^r$ | T. Charles |
| RmG479 | Ω5025::Tn*5*, *sfx-2* | Φ(Rm5348)->RmF346, $Nm^r$, from T. Charles |

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| RmG514 | 12 kb deletion of *fix114*, *sfx-2*, Ω5025::Tn*5* | T. Charles |
| RmG549 | ΔΩ5033-5064::Tn*5*-233, Ω5256::Tn*5* | Φ(BankGS2)->RmF346, $Nm^r$ $Gm^r$-$Sp^r$, deoxycholate resistant |
| RmG550 | ΔΩ5033-5064::Tn*5*-233, Ω5257::Tn*5* | Φ(BankNM1)->RmF346, $Nm^r$ $Gm^r$-$Sp^r$, deoxycholate resistant |
| RmG551 | ΔΩ5033-5064::Tn*5*-233, Ω5258::Tn*5* | Φ(BankNM1)->RmF346, $Nm^r$ $Gm^r$-$Sp^r$, deoxycholate resistant |
| RmG552 | ΔΩ5033-5064::Tn*5*-233, Ω5259::Tn*5* | Φ(BankNM1)->RmF346, $Nm^r$ $Gm^r$-$Sp^r$, deoxycholate resistant |
| RmG553 | ΔΩ5033-5064::Tn*5*-233, Ω5260::Tn*5* | Φ(BankNM1)->RmF346, $Nm^r$ $Gm^r$-$Sp^r$, deoxycholate resistant |
| RmG554 | ΔΩ5033-5064::Tn*5*-233, Ω5261::Tn*5* | Φ(BankNM1)->RmF346, $Nm^r$ $Gm^r$-$Sp^r$, deoxycholate resistant |
| RmG555 | ΔΩ5033-5064::Tn*5*-233, Ω5254::Tn*5*,*sfx-2* | Φ(RmG655)->RmF346, $Nm^r$, $Gm^r$-$Sp^r$, deoxycholate sensitive |
| RmG556 | ΔΩ5033-5064::Tn*5*-233, Ω5258::Tn*5*,*sfx-2* | Φ(RmG551)->RmF346, $Nm^r$, $Gm^r$-$Sp^r$, deoxycholate sensitive |
| RmG557 | ΔΩ5033-5064::Tn*5*-233, Ω5258::Tn*5*, *sfx-3* | Φ(RmG551)->RmG425, $Nm^r$, $Gm^r$-$Sp^r$, deoxycholate sensitive |
| RmG558 | Ω5258::Tn*5* | Φ(RmG551)->Rm1021, $Nm^r$ |

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| RmG559 | Ω5258::Tn*5* | Φ(RmG551)->Rm1021, $Nm^r$ |
| RmG560 | *pck-1*::Tn*V*, Ω5118::Tn*5*-233, *sfx-1* | Φ(Rm5439)->RmF994, $Nm^r$, $Gm^s$-$Sp^s$, $Succinate^-$ |
| RmG561 | *pck-1*::Tn*V*, Ω5118::Tn*5*-233, *sfx-1* | Φ(Rm5439)->RmF994, $Nm^r$, $Gm^s$-$Sp^s$, $Succinate^-$ |
| RmG588 | *ndvB* | J. Glazebrook |
| RmG590 | *exoD17*::Tn*5*-233 | J. Reed |
| RmG591 | *sfx-1* | Φ(Rm1021)->RmG560, $Succinate^+$, $Nm^s$, $Gm^s$-$Sp^s$ |
| RmG623 | RmG591, Ω6::Tn*phoA* | pTH56, Ω6::Tn*phoA* homogenotized into RmG591 |
| RmG624 | RmG591, Ω9A::Tn*phoA* | pTH56, Ω9A::Tn*phoA* homogenotized into RmG591 |
| RmG625 | RmG591, Ω9B::Tn*phoA* | pTH56, Ω9B::Tn*phoA* homogenotized into RmG591 |
| RmG626 | RmG591, Ω13::Tn*phoA* | pTH56, Ω13::Tn*phoA* homogenotized into RmG591 |
| RmG627 | RmG591, Ω20B::Tn*phoA* | pTH56, Ω20B::Tn*phoA* homogenotized into RmG591 |
| RmG628 | RmG591, Ω21A::Tn*phoA* | pTH56, Ω21A::Tn*phoA* homogenotized into RmG591 |
| RmG629 | RmG591, Ω21B::Tn*phoA* | pTH56, Ω21B::Tn*phoA* homogenotized into RmG591 |
| RmG635 | Ω5118::Tn*5*, *ndvB*::Tn*5*-233, *sfx-1* | Φ(RmG558)->RmF379, $Nm^r$, $Gm^r$-$Sp^r$ (Independent) |

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| RmG636 | Ω5118::Tn*5*, *ndvB*::Tn*5*-233, *sfx-1* | Φ(RmG558)->RmF379, $Nm^r$, $Gm^r$-$Sp^r$ (Independent) |
| RmG637 | Ω5118::Tn*5*, *ndvB*::Tn*5*-233, *sfx-2* | Φ(RmG558)->RmG479, $Nm^r$, $Gm^r$-$Sp^r$ (Independent) |
| RmG638 | Ω5118::Tn*5*, *ndvB*::Tn*5*-233, *sfx-2* | Φ(RmG558)->RmG479, $Nm^r$, $Gm^r$-$Sp^r$ (Independent) |
| RmG639 | Ω5262::Tn*5*-233, 26% linked to Ω5258::Tn*5* | Φ(BankGS2)->RmG558, $Nm^s$ $Gm^r$-$Sp^r$ |
| RmG640 | Ω5263::Tn*5*-233, 60% linked to Ω5258::Tn*5* | Φ(BankGS2)->RmG558, $Nm^s$ $Gm^r$-$Sp^r$ |
| RmG641 | Ω5264::Tn*5*-233, 28% linked to Ω5258::Tn*5* | Φ(BankGS2)->RmG558, $Nm^s$ $Gm^r$-$Sp^r$ |
| RmG655 | ΔΩ5033-5064::Tn*5*-233, Ω5254::Tn*5* | Φ(BankGS2)->RmF346, $Nm^r$ $Gm^r$-$Sp^r$, deoxycholate resistant |
| RmG656 | ΔΩ5033-5064::Tn*5*-233, Ω5255::Tn*5* | Φ(BankGS2)->RmF346, $Nm^r$ $Gm^r$-$Sp^r$, deoxycholate resistant |
| RmG657 | ΔΩ5033-5077::Tn*5*-233, *exoA31*, *sfx-1* | Φ(Rm7031)->RmF263, $Nm^r$ $Gm^r$-$Sp^r$ $Fix^-$ |
| RmG658 | ΔΩ5033-5064::Tn*5*-233, *exoA31*, *sfx-2* | Φ(Rm7031)->RmF346, $Nm^r$ $Gm^r$-$Sp^r$ $Fix^-$ |
| RmG659 | ΔΩ5033-5064::Tn*5*-233, *exoA31*, *sfx-3* | Φ(Rm7031)->RmG425, $Nm^r$ $Gm^r$-$Sp^r$ $Fix^-$ |
| RmG660 | ΔΩ5033-5077::Tn*5*-233, *exoB13*, *sfx-1* | Φ(Rm7013)->RmF263, $Nm^r$ $Gm^r$-$Sp^r$ $Fix^-$ |
| RmG661 | ΔΩ5033-5064::Tn*5*-233, *exoB13*, *sfx-2* | Φ(Rm7013)->RmF346, $Nm^r$ $Gm^r$-$Sp^r$ $Fix^-$ |
| RmG662 | ΔΩ5033-5064::Tn*5*-233, *exoB13*, *sfx-3* | Φ(Rm7013)->RmG425, $Nm^r$ $Gm^r$-$Sp^r$ $Fix^-$ |
| RmG663 | ΔΩ5033-5077::Tn*5*-233, *exoF55*, *sfx-1* | Φ(Rm7055)->RmF263, $Nm^r$ $Gm^r$-$Sp^r$ $Fix^-$ |

| | | |
|---|---|---|
| RmG664 | ΔΩ5033-5064::Tn*5*-233, *exoF55*, *sfx-2* | Φ(Rm7055)->RmF346, $Nm^r$ $Gm^r$-$Sp^r$ $Fix^-$ |
| RmG665 | ΔΩ5033-5064::Tn*5*-233, *exoF55*, *sfx-3* | Φ(Rm7055)->RmG425, $Nm^r$ $Gm^r$-$Sp^r$ $Fix^-$ |
| RmG666 | Ω5118::Tn*5*, Ω5122::Tn*5*-132 | Φ(RmF379)->RmG167, $Nm^r$, $Ot^r$ |
| RmG667 | Ω5118::Tn*5*-233, Ω5122::Tn*5*-132 | Φ(RmF994)->RmG167, $Gm^r$-$Sp^r$, $Ot^r$ |
| *Escherchia coli* | | |
| MM294A | *pro-82 thi-1 hsdR17 supE44 endA1* | Laboratory collection |
| MT607 | MM294A *recA56* | Finan et al. 1986 |
| MT614 | MT607ΩTn*5* | Finan et al. 1986 |
| MT616 | MT607(pRK600) | Finan et al. 1985 |
| MT620 | MT607 $Rf^r$ | T. Finan |
| MT621 | MM294A *malF*::Tn*phoA* | Yarosh et al. 1989 |
| DH5α | *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ*(argF-lacZYA)*U169 *ϕ*80d*lacZ*ΔM15 | B.R.L. Inc. |
| CB263-1 | Cosmid clone bank in MT607 using pRK7813 and RmF263 DNA, ca. 2400 pooled inserts | This work |
| CB263-2 | Cosmid clone bank in Mt607 using pRK7813 and RmF263 DNA, ca. 2250 pooled inserts | This work |

Plasmids

| | | |
|---|---|---|
| pRK2013 | ColE1 replicon with RK2 transfer region, Nm-Km$^r$ | Figurski and Helinski 1979 |
| pRK600 | pRK2013 *npt*::Tn*9*; Cm$^r$, Nm-Km$^s$ | Finan et al. 1986 |
| pRK607 | pRK2013::Tn*5*-233;Nm-Km$^r$, Gm$^r$, Sp$^r$ | DeVos et al. 1986 |
| pRK7813 | RK2 derivative carrying pUC9 polylinker and *cos* site, Tc$^r$ | Jones and Gutterson 1987 |
| pLAFR1 | Inc P cosmid cloning vector, Tc$^r$ | Friedman et al. 1982 |
| pTF1 | pBR322::Tn*V*; Ap$^r$, Nm-Km$^r$ | Furichi et al. 1985 |
| pTH21 | pLAFR1 clone carrying Rm1021 *fix114* | T. Charles |
| pTH23 | pLAFR1 clone carrying Rm1021 *exoZ* | T. Charles |
| pTH49 | pRK7813 clone carrying *RmF263 phe-232* | This work |
| pTH50 | pRK7813 clone carrying *RmF263 phe-232* | This work |
| pTH51 | pLAFR1 clone carrying Rm1021 *phe-502* | This work |
| pTH54 | pRK7813 clone carrying *sfx-1* | This work, isolated from CB263-1 |
| pTH55 | pRK7813 clone carrying *sfx-1* | This work, isolated from CB263-1 |
| pTH56 | pRK7813 clone carrying *sfx-1* | This work, isolated from CB263-1 |
| pTH57 | pRK7813 clone carrying *sfx-1* | This work, isolated from CB263-1 |

| | | |
|---|---|---|
| pTH60 | 7 kb *Hind*III fragment of pTH56 recloned into a *Hind*III deletion of pTH56 | This work |
| pTH61 | 12 kb *Hind*III fragment of pTH56 in pUC19 | This work |
| pTH62 | 2.2 kb *HindIII* fragment of pTH56 in pUC19 | This work |
| pPH1JI | IncP, $Gm^r$, $Sp^r$, $Cm^r$ | Beringer et al. 1978 |
| pGMI102 | $Nm^s$ derivative of RP4 | Meade et al. 1982 |
| pUC19 | Cloning vector, ColE1 *oriV*, $Amp^r$ | Yanisch-Perron et al. 1985 |
| *Phage* | | |
| ΦM1 | *R. meliloti* bacteriophage | Finan et al. 1985 |
| ΦM5 | *R. meliloti* bacteriophage | Finan et al. 1985 |
| ΦM7 | *R. meliloti* bacteriophage | Finan et al. 1985 |
| ΦM9 | *R. meliloti* bacteriophage | Finan et al. 1985 |
| ΦM10 | *R. meliloti* bacteriophage | Finan et al. 1985 |
| ΦM11 | *R. meliloti* bacteriophage | Finan et al. 1985 |
| ΦM12 | *R. meliloti* bacteriophage | Finan et al. 1985 |
| *Transposons* | | |
| Tn*5* | $Nm^r$, $Sm^r$ | Berg and Berg 1983 |
| Tn*5*-11 | *oriT* of pRK2 cloned into Tn*5*-233, $Gm^r$-$Sp^r$ | Finan et al. 1986 |
| Tn*5*-132 | $Ot^r$ | Berg and Berg 1983 |

| | | |
|---|---|---|
| Tn*5*-233 | $Gm^r$, $Sp^r$ | De Vos *et al.* 1986 |
| Tn*5*-235 | Tn*5* containing *E. coli lacZ*, $Nm^r$ | De Vos *et al.* 1986 |
| Tn*5*-*mob* | Tn*5* containing *mob* site from RK2 | R. Simon 1984 |
| Tn*V* | Tn*5* containing pSC101 *oriV*, $Nm^r$ | Furichi *et al.* 1985 |
| Tn*phoA* | Alkaline phosphatase fusion generating derivative of Tn*5*, $Nm^r$ | Manoil and Beckwith 1985. |

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Abbreviations are as follows: Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicn; Km, kanamycin; Nm, neomycin; Ot, oxytetracycline; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; *lac*, lactose utilization genes; *oriT*, origin of transfer; *oriV*, origin of vegetative replication; *mob*, mobilization region of RK2. (+) indicates clockwise *oriT* or *mob* transfer (ie. clockwise markers transferred early), (-) indicates counterclockwise *oriT* or *mob* transfer. ΦM12 transducing lysates are indicated by Φ preceeding the strain number. For strain constructions, an arrow indicates transduction from the indicated ΦM12 lysate into the recipient strain. Example: Φ(RmF338)->Rm1021, $Nm^r$, means a ϕM12 lysate grown on RmF338 was used to transduce $Nm^r$ into Rm1021. Δ5408 = ΔΩ5033-5077, ΔF114 = ΔΩ5033-5064.

[a] Unless otherwise indicated all strains are in a Rm1021 background and were constructed in this work.

was solidified with Difco Nobel agar (1.5% w/v). For R. meliloti $MgSO_4$ and $CaCl_2$ was routinely added to sterile LB broth, each to a final concentration 2.5 mM.

TY and YEM media were also used as complex media for R. meliloti; TY (Beringer 1974) consists of 5 tryptone, 3 g yeast extract, 3 mM $CaCl_2$, per litre of water and was solidified with 1.5% Difco Nobel agar. YEM (Vincent 1980) consists of 0.5 g $K_2HPO_4$, 0.2 g $MgSO_4$, 0.1 g NaCl, 10 g mannitol, 1.0 g yeast extract, 1.0 g $CaCl_2$ per litre of water and was solidified with 1.5% Difco Nobel agar.

Defined media for R. meliloti and E. coli was M9 salts media (Miller 1972). This contained 5.8 g $Na_2HPO_4$, 3.0 g $KH_2PO_4$, 0.5 g NaCl, and 1.0 g of $NH_4Cl$ per litre of water. $MgSO_4$, $CaCl_2$, biotin and a carbon source was added after autoclaving to a final concentration of 1 mM, 0.25 mM, 1.0 µg/ml and 15 mM respectively unless otherwise noted. This was solidified with 1.5% Difco Nobel agar.

1/2 GYM media (Dylan et al. 1990a) was used as a low osmolarity media for R. meliloti. This contained 0.05 mM glutamate (monosodium salt), 0.01% (w/v) yeast extract, 2.5 mM mannitol, 0.5 mM $K_2HPO_4$, and 0.25 mM $MgSO_4$. This media was also supplemented with either 100 mM or 500 mM NaCl.

Soft agar was used to plaque phage. This consisted of LB supplemented with $MgSO_4$ and $CaCl_2$ to a final

concentration of 2.5 mM and solidified with 0.7% (w/v) Difco Nobel agar.

LB/M9 agar (Charles et al. 1990) was used to plate out ΦM12 transductions. To make LB/M9 agar, 150 ml M9 salts (1X) were mixed with 150 ml LB agar containing 3% (w/v) Difco Nobel agar.

Yeast extract swarm media (Ames et al. 1980) contained 0.01% (w/v) yeast extract, 1.0 mM $MgSO_4$ per of litre water. This was solidified with 0.3% (w/v) Difco Nobel agar.

Jensen's media (Vincent 1980) was used for nodulations and contained 1.0 g $CaHPO_4$, 0.2 g $K_2HPO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl, 0.1 g $FeCl_3$, and was supplemented with 1.0 ml of 1000X trace elements solution (see below) per litre of water. The pH of the solution was adjusted to 7.0 with 1 M NaOH. For Jensen's agar the solution was solidified with 1% (w/v) Difco Nobel agar. Trace elements solution 1000X consisted of 1.0 g $H_3BO_3$, 1.0 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $CuSO_4 \cdot 5H_2O$, 0.5 g $MnCl_2 \cdot 4H_2O$, 1.0 g $NaMoO_4 \cdot 2H_2O$, 10.0 g EDTA, 2.0 g NaFeEDTA, and 0.4 g biotin per litre water.

Seeds were germinated on water agar media which consisted of water solidified with 1.5% (w/v) agar.

## Antibiotics and Indicators

The following antibiotics and concentrations were used to select for Tn*5*, Tn*5* derivatives, plasmids, and phenotypes

of bacterial strains used: 50 μg/ml ampicillin (Amp), 100 μg/ml bacitracin (Bac), 20 μg/ml chloramphenicol (Cm), 20 μg/ml gentamycin (Gm), 20 μg/ml kanamycin (Km), 100 μg/ml or 200 μg/ml neomycin (Nm), 50 μg/ml or 100 μg/ml spectinomycin (Sp), 100 μg/ml or 200 μg/ml streptomycin (Sm), 2 μg/ml, 5 μg/ml or 10 μg/ml tetracycline, and 0.5 μg/ml oxytetracycline.

To screen for inserts when cloning into pRK7813 or pUC19 the media was supplemented with 20 μg/ml 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-Gal). To visualize active fusions when mutagenizing with Tn*phoA* 20 μg/ml 5-bromo-4-chloro-3-indolyl phosphate was added to the media.

The following compounds were used to screen for relevant phenotypes of *R. meliloti*: 0.02% (w/v) calcofluor (pH of media was adjusted with 0.5 ml of 1 M NaOH with this addition), 1 mg/ml or 2 mg/ml filter sterilized sodium deoxycholate (Doc), 1 mg/ml deoxycholate and 0.25 mM EDTA (DE), 0.1 mg/ml sodium dodecyl sulfate (SDS), and 1 mg/ml n-lauroylsarcosine (sodium salt).

## Chemicals and Reagents

Antibiotics used for this work were obtained from Sigma or Boehringer Mannheim. Restriction endonucleases, $T_4$ ligase, DNA packaging extracts, and non-radioactive labelling and detection kit were all obtained from

Boehringer Mannheim. Other chemicals and reagents were all reagent grade and were obtained from Fisher Scientific Company, Difco Laboratories, Bio-Rad Laboratories, BDH Chemicals, or Sigma.

## Equipment

To pellet bacterial cells and DNA a Beckman GPR Tabletop centrifuge or a Sorvall RC-2 centrifuge was used. To CsCl band plasmid DNA a vTi65.1 rotor was used with a Beckman L8-70 ultracentrifuge and to fractionate DNA on a sucrose gradient a SW40.1 rotor was used with a Beckman L8-70 ultracentrifuge. Optical densities of cultures were measured at 675 nm in a Bausch & Lomb Spectronic 20.

# CHAPTER 3

# METHODS

## Plasmid Isolation

### Small scale isolation.

2.5 ml *E. coli* cultures carrying plasmids were grown overnight at 30°C in LB broth supplemented with a selective antibiotic. 1.5 ml was poured into an eppendorf tube and the bacterial cells were pelleted in a microfuge for 20 seconds, the supernatant decanted, and the pellet was resuspended in 350 µl STET (containing 8% w/v sucrose, 0.5% Triton X-100, 50 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0). To this, 10 µl lysozyme (50 mg/ml) was added and briefly vortexed. The tube was placed into a boiling water bath, the heat was turned off, and the tube was incubated for three minutes, and then centrifuged for 15 minutes at room temperature. The supernatant was then decanted to a sterile eppendorf tube, 350 µl isopropanol was added, mixed briefly, then placed at -70°C for 15 minutes. This was then spun in a microfuge at 5°C for 15 minutes. The supernatant was discarded and the pellet was washed first with 70% ethanol and then with 95% ethanol. The residual ethanol was removed by placing the pellet at 37°C for approximately fifteen minutes. The pellet was then resuspended in 50 µl of 20 mM

Tris pH 8.0, 1 mM EDTA pH 8.0 and heated to 65°C for fifteen minutes. This was stored at 5°C.

Large scale alkaline lysis plasmid isolation

Alkaline lysis plasmid preparations were done essentially as described by Maniatis et al. (1982) with some modifications.

E. coli strains were grown overnight in 250 ml LB cultures supplemented with the appropriate antibiotic. The cells were then transferred to centrifuge bottles and pelleted by spinning them at 6000 rpm for 15 minutes at 4°C with a GSA rotor. Cells were then resuspended in 20 ml TEG (containing 50 mM Tris HCl pH 8.0, 20 mM EDTA pH 8.0, 1% w/v glucose), 10 mg of lysozyme was added and the solution was briefly mixed. To this 40 ml of ALS (0.2 M NaOH, 1% SDS) was added and mixed. At this point the solution went clear and 90 ml of sterile water was added, mixed, then 30 ml of HSS (3 M potassium acetate pH 4.8) was added and the bottles were chilled at -70°C for fifteen minutes. This was then spun for 15 minutes at 6000 rpm at 4°C and then the supernatant was transferred through a single layer of cheese cloth into another sterile centrifuge bottle. 90 ml of isopropanol was added, mixed, and immediately spun for 15 minutes at 6000 rpm at 4°C. The supernatant was decanted and the pellet was dried at 37°C for approximately 15 minutes. The pellet was then resuspended in 9 ml of 50 mM

Tris HCl pH 8.0, 20 mM EDTA pH 8.0, and 9.9 gm CsCl was added. This solution was then transferred to 16x76mm (13.5 ml) Beckman polyallomer ultracentrifuge tubes. 0.5 ml of a 10 mg/ml ethidium bromide stock was added, the tube was topped with paraffin oil, heat sealed, then placed in a vTi65.1 rotor and centrifuged at 55,000 rpm at 10°C, for 18 hours. Plasmid bands were viewed using a hand held UV lamp and the plasmid band was removed using a 5 ml syringe and a 20g1 1/2 needle. Ethidium bromide was removed by extracting several times with isopropanol saturated with CsCl in water. The plasmid DNA was then precipitated by adding 2 volumes of water, 6 volumes of 95% ethanol and chilling at -20°C overnight or -70°C for approximately one hour. The DNA was pelleted by spinning the tubes at 5500 rpm at 5°C for 20 minutes in a Beckman tabletop centrifuge. The supernatant was removed, the pellet was dried at 37°C and finally resuspended in 200 $\mu$l 20mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0.

An alternate method that did not include CsCl banding was also used. This method is the same as outlined above except instead of resuspending in 9.0 ml of buffer containing CsCl the following steps were followed. The dried pellet of DNA was resuspended in 2.0 ml of 20 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0, RNA'ase was added to a concentration of 40 $\mu$g/ml and this was incubated at 37°C for

fifteen minutes. To this 2.0 ml of 13% PEG in 1.6 M NaCl was added and then incubated on ice for 30 minutes. The DNA was then pelleted by spinning the tubes for 15 minutes at 4000 rpm at 5°C in a tabletop centrifuge. The supernatant was discarded and the resulting pellet was resuspended in 0.5 ml of 20 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0, and extracted once with equilibrated phenol, once with chloroform/isoamylalcohol (24:1), ethanol precipitated, and resuspended in 200 µl of 20 mM Tris HC l pH 8.0, 1 mM EDTA pH 8.0.

## Total DNA isolation

Total DNA isolation was essentially as described by Meade (Meade *et al.* 1982) with minor modifications. 2.5 ml cultures of *R. meliloti* was grown overnight in LBmc. Cells were pelleted and washed once in 5.0 ml of 0.85% saline, once with TES (10 mM Tris HCl pH 8.0, 25 mM EDTA pH 8.0, 150 mM NaCl), once with 10 mM Tris HCl pH 8.0, 25 mM EDTA pH 8.0, containing 0.1% (w/v) n-lauroyl-sarcosine, and finally resuspended in 2.5 ml 10 mM Tris HCl pH 8.0, 25 mM EDTA. 0.25 ml of lysozyme (2 mg/ml) was added, mixed, and incubated for 15 minutes. 0.3 ml of sarkosyl/protease (5 mg/ml predigested Pronase, 10% n-lauroyl-sarcosine in 10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0) was added and was incubated for approximately one hour at 37°C. When complete

lysis was apparent the solution was extracted once with an equal volume of phenol followed by chloroform/isoamylalcohol (24:1) extractions until there was no material left at the interface. The solution was adjusted to 0.3 M ammonium acetate and 0.54 volumes of isopropanol was added. The two phases were gently mixed by swirling the tube. The precipitated DNA was removed with a pasteur pipette and the DNA was washed first with 70% ethanol followed by a 95% ethanol wash. The DNA was then dissolved in 0.5 ml of 20 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0.

## Restriction analysis

Restriction of plasmid or total DNA was done in a volume of 20-50 $\mu$l containing between 100-500 ng of DNA at 37°C for 1 to 4 hours. Restriction reactions generally contained between 1-5 units of enzyme and the appropriate buffer as recommended by the manufacturer. RNA'ase was added to final concentration of 0.05 $\mu$g /$\mu$l to small scale plasmid preps.

Restriction fragments were separated on 0.8-1.0% agarose gel using either TAE (containing 40 mM Tris HCl, 20 mM sodium acetate, 2 mM EDTA, pH 8.0 with glacial acetic acid) or TBE (Maniatis et al. 1982) running buffer. Fragments were stained with ethidium bromide (5 $\mu$g/100 ml

staining volume) for at least 40 minutes, briefly destained with water and were visualized using a UV light source.

Isolation of DNA from low melting point agarose gels

Plasmid DNA (approximately 5 μg) that contained the fragment of interest was restricted in a 100 μl volume with excess restriction enzyme for 1 hour. This was loaded onto a 0.8% TAE agarose gel such that the two flanking lanes of the gel were loaded with 1/25 of the restriction volume and the remaining volume was distributed evenly among 3 intervening lanes. After electrophoresis, the flanking lanes were cut from the gel and stained with ethidium bromide. The gel was reconstructed on a UV light box and the band of interest was cut from the unstained portion of the gel. The gel slice was then transferred to a 50 ml Falcon tube, 5 volumes of 50 mM Tris HCl pH 8.0, 20 mM EDTA was added and heated to 68°C for 20 minutes. This solution was extracted once with an equal volume equilibrated phenol, twice with chloroform/isoamylalcohol (24:1), ethanol precipitated, and resuspended in 50 μl of 20 mM Tris HCl pH 8.0, 1 mM EDTA.

Ligation reactions

Ligations were generally carried out in 10 μl volumes. DNA to be ligated was mixed together in an eppendorf tube in

a ratio of approximately 2:1 (insert:vector), ethanol precipitated, and resuspended in ligation buffer (containing 66 mM Tris HCl pH 7.5, 6.6 mM $MgCl_2$, 10 mM dithiothreitol). This was briefly heated to 65°C then ATP and $T_4$ ligase were added to a concentration of 1 mM and 0.1 unit/$\mu$l respectively. Volumes were then adjusted to 10 $\mu$l with sterile $ddH_2O$. Ligations were incubated at 12°C for 12-18 hours. Ligation was confirmed by running an aliquot on a agarose gel.

Competent cell preparation and transformation

Competent cell preparation was carried out according to the procedure described by Ausubel et al. (1987). A 4 ml culture of DH5$\alpha$ was grown overnight at 30°C and used to inoculate 400 ml of LB broth in a 2 l flask. The culture was incubated at 37°C with shaking (200 rpm) and grown to an optical density of 0.4 ($\lambda$=590 nm). Cells were then transferred to prechilled 50 ml Falcon tubes and centrifuged for 7 minutes at 3300 rpm at 5°C in a Beckman GPR tabletop centrifuge using a GA 10 rotor. Pellets were gently resuspended in ice cold $CaCl_2$ solution (containing 60 mM $CaCl_2$, 15% w/v glycerol, 10 mM MOPS pH 7.0, filter sterilized) then pelleted for 5 minutes at 2600 rpm (1000xg) at 5°C. The supernatant was decanted and the cells were again resuspended in 40 ml of ice cold $CaCl_2$ solution,

incubated on ice at 5°C for 30 minutes, and pelleted for 5 minutes at 2600 rpm (1000xg) at 5°C. The cells were then resuspended in 8 ml of ice cold $CaCl_2$ solution and incubated overnight on ice at 5°C. The cells were then aliquoted into eppendorf tubes, quick frozen by pouring liquid nitrogen over the tubes and stored at -70°C until needed.

Competent cells were transformed by adding 10-40 ng of DNA (in 20 mM Tris pH 8.0, 1 mM EDTA) to 100 $\mu$l of competent cells. These were incubated together on ice for 30 minutes, heat shocked by placing them into a 42°C water bath for 2 minutes, then placed back on ice for 3 minutes. To this 1 ml of prewarmed (37°C) LB was added and the cells were incubated at 37°C for at least 1 hour (phenotypic lag period). The cells were then pelleted for 20 seconds in a microfuge, resuspended in 110 $\mu$l of LB broth and a $10^0$ and a $10^{-1}$ dilution were plated on the appropriate selective media. The plates were then incubated at 37°C overnight.

## Cosmid cloning

RmF263, containing the sfx-1 allele in a Δ5408 background, was grown overnight in 5 ml culture in LBmc. This was used to inoculate 250 ml of LB broth in a 1000 ml flask and was grown overnight at 30°C.

Cultures were pelleted by spinning at 6000 rpm using a GSA rotor. Cells were washed once with 50 ml of 20 mM Tris

HCl pH 8, 20 mM EDTA pH 8, 150 mM NaCl, once with 50 ml of 20 mM Tris HCl pH 8, 20 mM EDTA pH 8, 1% sarkosyl, and finally resuspended in 22 ml of 20 mM Tris HCl pH 8, 20 mM EDTA pH 8.

Cells were lysed by adding lysozyme to a concentration of 0.5 mg/ml incubating for fifteen minutes at 37°C then adding predigested pronase/sarkosyl to final concentration of 0.5 mg/ml pronase 1% sarkosyl. This was incubated at 37°C for 2 hours.

The cell lysate was extracted twice with an equal volume of equilibrated phenol and twice with chloroform/isoamylalcohol (24:1). The DNA was then precipitated by adding ammonium acetate to 0.3 M and adding approximately 0.5 volumes of isopropanol. The mixture was swirled very gently to bring the nucleic acids out of solution. The DNA was taken out with a pasteur pipette, washed with 70% ethanol, redissolved in 15ml of 20 mM Tris HCl pH 8, 20 mM EDTA pH 8, extracted once with chloroform/isoamyl-alcohol and reprecipitated with ammonium acetate and isopropanol. The pellet was washed with 70% ethanol followed by a 95% ethanol wash. The residual alcohol was drained from the pellet and then placed in 3.0 ml of 20 mM Tris HCl, 1 mM EDTA pH 8, heated to 65°C for 15 minutes and then allowed to go into solution at 5°C . The

quality and quantity of the DNA was checked by running an aliquot on a 0.5% TAE agarose gel.

## Partial Digestion of Genomic DNA

50 μg of genomic DNA was digested in a 1 ml volume at 37°C with varying amounts of BamH1 (25 units, 12 units, 6 units, 0 units) per restriction. One third of each restriction was removed at 45 minutes, 60 minutes, and at 90 minutes. The reactions in the aliquots that were removed was stopped by adding EDTA to a concentration of 20 mM and placing the samples on ice. The samples from each concentration were pooled, phenol extracted, ethanol precipitated, and resuspended in a 500 μl volume of 20 mM Tris HCl pH 8, 1 mM EDTA pH 8. An aliquot from each different enzyme concentration was analyzed for the extent of digestion by running an aliquot on a 0.6% TAE agarose gel.

## Size Fractionation of Partial Digests

Partially-digested DNA was fractionated on a 10%-40% (w/w) sucrose gradient made up in 20 mM Tris HCl pH 8.0, 20 mM EDTA pH 8.0, 1 M NaCl. The gradients were poured in 10% increments into sterile 14x95mm Beckman polyallomer tubes and allowed to diffuse for 4-5 hours at room temperature. 50-100 μg of partially digested genomic DNA in a 500 μl volume was layered onto the top of a 10%-40% gradient and

then centrifuged at 17°C, 23K rpm for 18 hours using a SW40.1 rotor. Gradients were then fractioned by puncturing the bottom of the tube with a syringe and collecting one ml aliquots. Aliquots were then diluted with one volume of 20 mM Tris HCl pH 8, 1 mM EDTA pH 8 and precipitated by adding 2 volumes of 95% ethanol and chilling at -70°C for 30 minutes. DNA was pelleted by spinning in a Beckman table top centrifuge at 5°C, 5500 rpm, for 20 minutes. The supernatant was decanted and the pellet was washed with 70% ethanol followed by a 95% ethanol wash. Residual ethanol was removed by drying the pellet at 37°C. The pellet was resuspended in 200 $\mu$l and 30 $\mu$l aliquots were analyzed by electrophoresis on a 0.6% agarose gel to check for size distribution. Size markers were as follows: pTH21 digested with HindIII giving fragments of 30.7 kb, 7.8 kb, 5.1 kb, pTH21 digested with ClaI giving fragments of 41.9 kb and 1.6 kb, and lambda digested with HindIII. Fractions that had DNA in the 20-40 kb range were pooled, ethanol precipitated, and resuspended in 50 $\mu$l of 20 mM Tris HCl pH 8, 1 mM EDTA pH 8.0 and stored at 5°C.

## Vector Preparation

pRK7813 (Jones and Gutterson 1987) was used as a cloning vector. 10 $\mu$g of CsCl purified pRK7813 was digested

with 40 units of BamHI for one hour at 37°C. The sample was then extracted once with equilibrated phenol, once with chloroform/isoamylalcohol (24:1), ethanol precipitated, and resuspended to a final concentration of 0.5 μg/μl. The condition of the cut vector was checked by analyzing an aliquot on a 0.8% TAE agarose gel.

## Vector-Insert Ligation

Ligation of BamHI cut, size fractioned insert to BamHI cut pRK7813 was carried out in a 10 μl volume at a high DNA concentration (0.3 μg/μl) to promote concatamer formation (Maniatis et al. 1982). Several vector:insert ratios (10:1, 5:1, 2:1, 1:2) were tried to optimize concatamer formation. Vector DNA and insert DNA were mixed together, ethanol precipitated, and resuspended in ligation buffer. Ligations were carried out for 18 hours at 12°C. 1 μl of each ligation was checked by gel electrophoresis for successful ligation.

## Invitro Packaging of Ligated DNA

Ligated vector/insert DNA was packaged using a Boehringer Mannheim DNA Packaging Kit according to the manufacturer's protocol. The packaging reaction utilizes freeze thaw lysate (FTL) and a sonic extract (SE). Each

lysate is provided from strains harbouring a defective lambda prophage that are incapable of producing a viable phage particle but are capable of packaging exogenous DNA if it carries lambda cos sequences (Hohn 1979).

5 µl of FTL was placed into a prechilled eppendorf tube on ice. To this, 1.5 µl of ligated vector/insert DNA (approx. 0.15 µg) was added. Finally 7.5 µl of SE was added, the mixture was gently mixed with a pipetteman tip and incubated for one hour at room temperature.

The packaging mix was then diluted ten fold with lambda buffer containing 10 mM Tris HCl, 10 mM $MgSO_4$, 0.01% gelatine (w/v), pH 7.4 and sterilized using 25 µl chloroform. The lysate was then decanted to a sterile eppendorf and stored at 5°C.

## Titering Lysates

Lysates were diluted 1/10 and 1/50 in lambda buffer. MT607 was grown to saturation in LB broth containing 2% maltose (w/v). Cells were pelleted and resuspended in 0.4 volumes of 10 mM $MgSO_4$ and aerated for several hours at 30°C. Equal volumes (25 µl) of lysate and cells were mixed and incubated at room temperature for 30 minutes. At this time 200 µl of prewarmed (37°C) LB broth was added and the transductions were incubated at 37°C for a further 45 minutes to phenotypic lag. Transductions were then

pelleted, resuspended in 100 µl of LB broth and plated onto LB agar containing tetracycline (10 µg/ml) and incubated at 37°C. Plates were scored after 18 hours of incubation.

Large Scale Transfection and Freezing of Cosmid Bank

Large scale transfection was carried out in the same manner as described above except that volumes were four times greater. All colonies that were on the selective plates were pooled into two independent cosmid banks. These were then grown up to mid-log phase (O.D. approx. 0.7-0.8 at λ=675nm) in LB broth containing 5 µg/ml tetracyline. This culture was aliquotted and stored as a frozen permanent at -70°C.

Southern blotting and hybridization

Southern blotting was carried out as described by Southern with modifications (Maniatis et al. 1982). DNA was first cut with the appropriate enzyme and separated on a TAE agarose gel. After first staining the gel with ethidium bromide, the gel was rinsed with distilled water then soaked in 0.25 M HCl for 10 minutes to depurinate the DNA. The gel was then washed twice for 15 minutes in 0.5 M NaCl, 150 mM NaOH to neutralize the gel and to denature the DNA, rinsed

with distilled water and soaked in 0.5 M Tris HCl, 0.5 M NaCl pH 7.4 for 30 minutes. DNA was then transferred to a nylon membrane by capillary action using 20X SSC (175.3 g NaCl, 88.2 g sodium citrate, pH to 7.0, / litre water). Transfer was carried out for 12-18 hours. Nylon filters were then baked at 80°C for at least 2 hours.

## Non-radioactive labelling of probes

A random priming method of DNA synthesis was used to incorporate digoxigenin labelled dUTP into the desired DNA probe. 1 μg of DNA in a volume of up to 5 μl was denatured by heating the sample to 95°C for 10 minutes then quickly cooling it in an ice/alcohol bath for 3 minutes. To this 2 μl of random hexanucleotides, 2 μl dNTPs containing digoxegenin labelled dUTP, and 2 units of Klenow polymerase were added to the DNA sample. The volume was then made up to 20 μl and the reaction was incubated at 37°C overnight. The reaction was stopped by adding 2 μl of 0.2 M EDTA and then precipitated by adding 2.5 μl of 4 M LiCl and 75 μl of ice cold 95% ethanol. This was placed at -70°C for 30 minutes and pelleted using a microfuge for 20 minutes at 5°C. The supernatant was decanted, the DNA pellet was washed with 95% ethanol, and the residual ethanol was removed by placing the sample at 37°C . The labelled DNA

was then resuspended in 50 $\mu$l of 20 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.

## Hybridization

Nylon filters were first prehybridized for at least 1 hour at 42°C in a small plastic container with at least 20 ml prehybridization solution containing 5X SSC, 50% (v/v) deionized formamide, 0.1% (w/v) n-lauroyl-sarcosine, 0.02% (w/v) SDS, and 5.0% (w/v) blocking reagent.

To hybridize, the prehybridization solution was removed and 1.5 ml/100 $cm^2$ hybridization solution which consisted of prehybridization solution with 5-10 $\mu$l of labelled probe, was added to the nylon membrane. Hybridizations were carried out for 12-16 hours at 42°C.

After hybridization the labelled probe was removed and the filters were washed 2 X 5 minutes with 2X SSC, 0.1% (w/v) SDS at room temperature followed by 2 X 15 minute washes with 0.2X SSC, 0.1% (w/v) SDS at 68°C.

## Detection

The detection protocol was that which was outlined with the non-radioactive labelling kit. Filters were washed for one minute in buffer 1 (containing 100 mM Tris-HCl, 150 mM NaCl, ph 7.5) then blocked for at least 30 minutes using approximately 100 ml 0.5% (w/v) blocking reagent

(manufacturer supplied) made up in buffer 1. The filter was then washed once with buffer 1, and then treated for 30 minutes with 30 ml anti-digoxigenin antibody (supplied with kit) diluted 1/5000 in buffer 1. Filters were then washed twice (15 minutes each) with buffer 1 to remove unbound antibody, and then equilibrated for 2 minutes in 40 ml buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). Buffer 3 was removed from the filters and detection was carried out by adding 20 ml of colour solution which consisted of 4-nitrobluetetrazolium chloride 169 μg/ml, and 5-bromo-4-chloro-3-indolyl phosphate (X-phos) 8.5 μg/ml in buffer 3. Colour reactions were carried out overnight at either room temperature or 37°C. Colour reactions were terminated with the addition of a stop solution containing 20 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0.

Isolation of second site symbiotic suppressors

Second site suppressors to fix114 were isolated by isolating $Fix^+$ nodules from alfalfa plants 23 days after they were inoculated with either RmF114 or Rm5408. $Fix^+$ nodules were then surface sterilized by first treating the nodule with 1% (v/v) hypochlorite, for one minute, followed by thorough washing with sterile distilled water. The nodules were then placed in 0.5 ml LB broth containing 0.3 M sucrose and crushed using a sterile inoculating stick.

Bacteria were streaked from this onto LB agar. Single colony isolates were tested for the original markers and reinoculated on alfalfa to confirm that the nodule isolates were $Fix^+$.

## Generalized transduction

General transduction was performed using ΦM12 (Finan et al. 1984). Lysates were made by growing up the donor strain overnight in a 5 ml broth culture of LBmc. The overnight culture was diluted to an O.D. of approximately 0.4 ($\lambda$= 675 nm) in 5 ml LBmc. Approximately 0.05-0.1 ml of a ΦM12 lysate grown on wild type strain was added and the culture was incubated on a roller shaker for 8-12 hours. Lysates were then treated with one drop of chloroform, the supernantant decanted from the cell debris and stored at 5°C until needed.

The recipient strain was grown overnight in LBmc to late log phase. Equal volumes (1 ml) of the donor lysate, diluted 1/30 in LBmc, and the overnight recipient were mixed and incubated for 20 minutes at room temperature. 2.5 ml 0.85% saline was then added and the cells were pelleted in a bench top centrifuge. The cells were then washed with 2.5 ml 0.85% saline, pelleted and resuspended in 0.5 ml 0.85% saline. 0.1 ml was then plated on LB/M9 agar supplemented with the appropriate antibiotics. Plates were then

incubated for 3-4 days at 30°C. Controls consisting of 0.1 ml of the diluted donor lysate and the recipient strain were also plated on the same selective media.

## Triparental matings

Plasmids (*tra*$^-$, *mob*$^+$) were routinely moved into strains by conjugation utilizing pRK600 as a mobilizing plasmid. Triparental matings were done either by growing up all parents in broth culture or by patch mating colonies from agar plates.

For patch mating plasmids, recipient, donor, and mobilizer colonies were transferred and spread in a 1 $cm^2$ area with a sterile toothpick from a fresh agar culture supplemented with the appropriate antibiotic onto a LB agar plate, incubated overnight at 30°C, and streaked onto selective media.

Alternatively, recipient, donor, and mobilizer cultures were grown overnight in LB broth with an appropriate antibiotic to maintain plasmid presence. Mobilizer and donor cultures were mixed in equal volume, pelleted, and resuspended in 0.85% saline. Equal volumes of recipient and mobilizer/donor cultures were mixed and about 0.05 ml was placed as a drop onto an LB plate, dried, and incubated overnight at 30°C. Mating spots were then either streaked out or resuspended in 0.85% saline and spread onto plates

that contained an antibiotic which selected for the plasmid (normally Tc) and another antibiotic to counterselect the donor and mobilizer strain (normally Sm).

Chromosomal mapping utilizing Tn5-mob mapping strains

A series of strains containing Tn5-mob linked to different auxotrophic markers in Rm1021 were constructed (Klein 1987). The positions of the Tn5-mob insertions are such that the entire chromosome could be mobilized in sections. Tn5-233 insertions were mapped by first transducing the insertion into the Tn5-mob mapping strains. Each of these transductants were then mated with Rm5000 using pGMI102 as a mobilizing plasmid. $Rf^r$, $Gm^r$, $Sp^r$ recombinants were scored as number of recombinants / donor.

Isolation of inserts linked by transduction

To isolate inserts linked by transduction to any other given marker the method described by Finan et. al. (1985) was followed. A lysate of a random transposon insertion bank in Rm1021 (for Tn5, and Tn5-132) or Rm5000 (for Tn5-233) was employed to transduce an appropriate antibiotic resistance into the strain carrying the target marker to which a linked insert was desired. Transductants were then

screened for loss of the target marker and those which had lost the marker were single colony purified at least two times. Transductional linkage was then determined by transducing the antibiotic resistance from the purified strains into a recipient strain carrying the original target marker.

## Replacement of Transposons

Replacement of transposon inserts was performed as previously described (Finan *et al.* 1986). To replace Tn*5* with Tn*5*-233 the recipient culture (containing Tn*5*) was grown up overnight in LBmc. A donor strain (*E. coli* MT607) containing Tn*5*-233 on plasmid pRK607 was mated with the recipient *R. meliloti* strain. Matings were incubated overnight at 30°C and plated onto LB agar containing Sm 400 µg/ml, Gm 20 µg/ml, and Sp 100 µg/ml. Resultant colonies were screened for loss of Tn*5* and the presence of Tn*5*-233. Putative replacements were tested for linkage to other markers in the area.

To replace Tn*5*-132 markers with TnV the same protocol was used except the plasmid containing TnV was pTF1 and selection was on LB agar containing Sm 200 µg/ml, Nm 200 µg/ml.

## Isolation of cosmids containing sfx-1 from root nodules

The fix114 suppressor mutation in RmF263 was designated sfx-1. To isolate cosmids that contained the sfx-1 allele two cosmid banks carrying insert DNA from RmF263 were conjugally mated into Rm5408 and RmF114. Between 14,000 and 31,000 $Sm^r$ $Tc^r$ transconjugants were pooled and inoculated onto 5 day old alfalfa seedlings. After 20-21 days vigorously growing green plants were uprooted and pink $Fix^+$ nodules were removed, surface sterilized, squashed in LB broth containing 0.3 M sucrose, either individually or in groups of 10 and spread onto LB containing Tc 10 μg/ml. Single colonies were then isolated and the cosmids were mated into E. coli (MT607) for restriction analysis. Representative cosmids were mated into strains which contained ΔF114 or Δ5408. The resultant constructs were then tested for symbiotic suppression by inoculating alfalfa and scoring for a reversal of the $Fix^-$ phenotype.

## Tn5 mutagenesis of pTH56

pTH56 was transferred from MT607 into MT614 using pRK600 as a mobilizing plasmid. Ten independent cultures of MT614/pTH56 were grown overnight in LB broth containing Tc (5 μg/ml) and mixed with an equal volume (2.5 ml) of MT616 grown up in LB broth with Cm (10 μg/ml). This was immediately pelleted and resuspended in 2.5 ml LB broth.

0.1 ml of an overnight culture of R. meliloti RmG490 was mixed with 0.1 ml of the donor/mobilizer in an eppendorf tube. To reduce volume this was pelleted, resuspended in 0.1 ml LB broth, and spotted onto LB agar plates and incubated overnight at 30°C.

Mating spots were resuspended in 2.5 ml of 0.85% saline and 0.1 ml was plated onto LB agar containing Sm 50 μg/ l, Nm 100 μg/ml, Tc 2 μg/ml. Resultant transconjugants were screened directly for loss of suppression on low osmolarity media.

## TnphoA mutagenesis of pTH56

pTH56 was transferred into MT621 using pRK600 as a mobilizing plasmid. Ten independent cultures of MT621/pTH56 were grown to late log phase in LB containing Tc (5 μg/ml). A mobilizer culture, MT616, was grown to late log phase in LB broth containing Cm (10 μg/ml). Mobilizer and donor cultures were mixed together in equal volumes (2.5 ml), immediately pelleted, and resuspended in 2.5 ml of LB broth. 0.1 ml of an overnight culture of R. meliloti RmF222 was mixed with 0.1 ml of the mobilizer/donor in an eppendorf. To reduce volume this was pelleted, resuspended in 0.1 ml of LB broth, spotted onto LB agar, and incubated at 30°C overnight.

Mating spots were resuspended in 0.5 ml of 0.85% saline and 0.1 ml of each resuspension was spread onto LB containing Sm (200 μg/ml), Nm (100 μg/ml), Tc (2 μg/ml), X-Phos (60 μg/ml). Plates were incubated for 3-5 days and blue colonies were isolated and single colony purified. Plasmids that contained putative TnphoA fusions were mated into MT607 for subsequent restriction analysis.

## Homogenotizations

Homogenotizations were carried out essentially as described by Ruvkin and Ausubel (1981). Plasmids containing Tn5 or TnphoA were mated into R. meliloti RmG591 and purified on LB SmTc (200/10 μg/ml) agar. Single colony isolates were grown overnight in LB broth containing Nm (50 μg/ml) and were mixed with an equal volume (1 ml) of log phase culture of E. coli J53/pPH1JI. This was pelleted, resuspended in 0.2 ml of 0.85% saline and 0.1 ml was spotted on LB agar and incubated overnight at 30°C. Mating spots were resuspended in 5 ml 0f 0.85% saline and $10^0$ and $10^{-1}$ dilutions were plated on LB agar containing Sm (100 μg/ml), Nm (100 μg/ml), Gm (70 μg/ml). Plates were incubated at 30°C and colonies were single colony purified at least twice. Homogenotes were confirmed by Southern analysis or by transductional linkage to other markers.

## Phage phenotype

Phage phenotype was determined in the following manner: Rhizobium meliloti strains to be tested were grown overnight in LBmc broth cultures and 0.1 ml was added to 1 ml of molten LBmc soft agar (50°C), vortexed briefly, and poured on LBmc agar plates and allowed to set. 10 µl of phage stocks were spotted onto the soft agar and the plates were incubated overnight at 30°C. Plates were scored after 24 hours.

## Motility assay for R. meliloti

To assay motility differences of R. meliloti strains, three independent cultures of each strain were grown overnight in LBmc. 10 µl of an overnight culture was spotted onto the center of a yeast extract swarm plate. Plates were then incubated at 30°C and scored at approximately 24 hour intervals. Results were then expressed as the mean swarm diameter at a given time.

## Nodulation Assays

Plant nodulation tests were carried out in Leonard jar assemblies (Vincent 1980) using quartz sand:vermiculite

mixture (1:1) watered with sterile distilled water and nitrogen-free Jensen's media (Vincent 1980).

Seed preparation: Seeds were surface sterilized in the following manner. They were weighed out (approximately 0.25 g/100 seeds) or roughly counted, and placed in a flask. Seeds were then treated with 95% ethanol for 20 minutes, 30 minutes with 2.5% hypochlorite, washed with at least 10 volumes (relative to the hypochlorite) of sterile distilled water over the period of at least one hour, and aseptically spread out on water agar plates and allowed to germinate in the dark for 2-3 days at room temperature.

Preparation of Leonard jar assemblies: Leonard jar assemblies consisted of autoclavable plastic pots with a 1-2 cm drainage hole in the bottom and a 250 ml beaker which could support the pot. A wick made of cotton batting was placed into the drainage hole. Over this, a wad of cotton was placed into the bottom of the pot over the wick. This was then placed into a beaker and a 1:1 sand/vermiculite mixture was added to the pot and watered with 250 ml of Jensen's solution. The joint between the jar and the beaker and the top of the jar was wrapped with aluminum foil and the assembly was autoclaved for at least 2 hours.

Planting and maintenance of plants: Ten 2-3 day old seedlings were aseptically transferred from water agar plates to Leonard jar assemblies and covered with

approximately 0.5-1.0 cm of sterile sand/vermiculite. The jars were then placed into environmental chambers with a 16 hour 22°C day, and an 8 hour 19°C night. Plants were inoculated on the fifth day following germination with an overnight culture of R.meliloti (O.D. 0.8-1.0, wavelength 570 nM) diluted 1/100 in 10 ml of sterile distilled water. Plants were watered with sterile distilled water as required. Alfalfa was generally grown for 23 days and sweet clover was grown for 25-28 days after inoculation before phenotypes were scored.

Determination of effectiveness: The symbiotic phenotype was scored by at least one of three methods: detaching the root systems and assaying by acetylene reduction, detaching the shoots and leaves from the roots to determine how much nitrogen has been fixed by dry weight analysis, or by scoring the plants visually by comparing with the control plants.

Acetylene reduction assay

The sand/vermiculite mixture covering the roots and nodules of the plants was removed by tipping a pot, separating the plants and the root systems, and then placing 3 plants into a 18 X 150 mm test tube. Nine plants were usually assayed per strain. The tops of the plants were removed, the test tube was sealed with a serum stopper. Three ml of acetylene (10% total atmosphere) was injected

and the excess pressure was released. Plant tops were removed at most 5 minutes before the start of the assay. Root systems were incubated for 15 minutes at room temperature, 0.3 ml was removed from the test tube and analyzed for ethylene production using a Pye Unicam gas chromatograph with a flame ionization detector and a 5'x 25''O.D. Porapak N 80/100 column. Peaks were standardized by comparing peaks to a standard containing 7.54 nM ethylene (0.1 ml of 700 ppm ethylene in $N_2$ gas). Activities were presented as nM ethylene produced per plant per hour.

Dry weight analysis

Dry weights were used to determined the cumulative nitrogen accumulation during a nodulation experiment. The tops of the plants were clipped just below the cotyledon of the plants after 23 days (alfalfa) or 28 days (sweet clover). All the plants from each pot were collected, placed in a paper bag, and dried at 75°C for at least one week. At this time the contents of each bag was weighed and the weights were expressed as mg / plant.

## Nodulation kinetics

Nodulation kinetics were carried out as described by Vincent (1980). 10 ml of Jensen's media was solidified with 1% agar and tubes were slanted and allowed to solidify. Alfalfa seeds were surface sterilized, germinated, and two

day old alfalfa seedlings were aseptically transferred to the Jensen's slants (one seedling per tube). Slants containing seedlings were placed into an environmental chamber at this time and five day old seedlings were inoculated with 0.1 ml of a 1/100 dilution of an overnight culture (O.D. approx. 0.8-1.0 wavelength 570 nM) diluted in water. For each strain 25 plants were inoculated with 5 independent cultures (ie. 5 plants/independent culture). The number of nodules per plant was scored every other day after inoculation starting on day 3 until day 29. Results were expressed as percentage of plants nodulated and the number of nodules per plant.

# CHAPTER 4

## MAPPING AND CLONING OF THE sfx-1 MUTATION

### Second site suppressors reverse the symbiotic phenotype of strains carrying ΔF114 or Δ5408

Pink Fix$^+$ nodules occasionally arose on roots that were inoculated with either Rm5408 or RmF114. Strains Rm5408 and RmF114 induced white Fix$^-$ nodules on alfalfa. Occasionally however, pink Fix$^+$ nodules arose on plants inoculated with these strains. Bacteria were isolated from these nodules and five such isolates obtained from independent nodulation experiments were further examined. These strains were designated RmF263, RmF346, RmG203, RmG204, and RmG425. When these five isolates were reinoculated onto alfalfa all were Fix$^+$ as determined by dry weight analysis of inoculated plants (Table 2). All five isolates appeared to carry the original deletion since all strains retained the Tn*5*-233 resistance determinants (Gm$^r$-Sp$^r$), were dark on calcofluor plates, and were unable to utilize dulcitol as a sole carbon source. The latter two markers were determined to be within ΔF114 and Δ5408 but not associated with *fix114* (T. Charles personal communication).

TABLE 2. DRY WEIGHTS OF ALFALFA INOCULATED WITH STRAINS CARRYING SECOND SITE REVERTANTS

| Strain | Relevant Characteristics | Dry Wt. (mg/plant) |
|---|---|---|
| Rm1021 | wild type | 25.8 |
| Rm5408 | Δ5408 | 5.5 |
| RmF114 | ΔF114 | 6.7 |
| RmF263 | Δ5408, *sfx-1* | 26.2 |
| RmG203 | Δ5408, *sfx-4* | 36.9 |
| RmG204 | ΔF114, *sfx-5* | 24.8 |
| RmF346 | ΔF114, *sfx-2* | 21.1 |
| RmG425 | ΔF114, *sfx-3* | 25.2 |
| Uninoculated | | 5.2 |

Shoot dry weights were determined on twenty three day old alfalfa plants. Values represent the mean of 17-19 plants.

To explain these results we hypothesized that the five Fix$^+$ isolates carried second site mutations which suppressed the Fix$^-$ phenotype associated with Δ5408 and ΔF114. To test this hypothesis further, more detailed characterization was carried out on strain RmF263. RmF263 was a Fix$^+$ isolate obtained from a pink nodule on alfalfa roots inoculated with Rm5408.

Second site mutation in RmF263 is not linked to Fix114

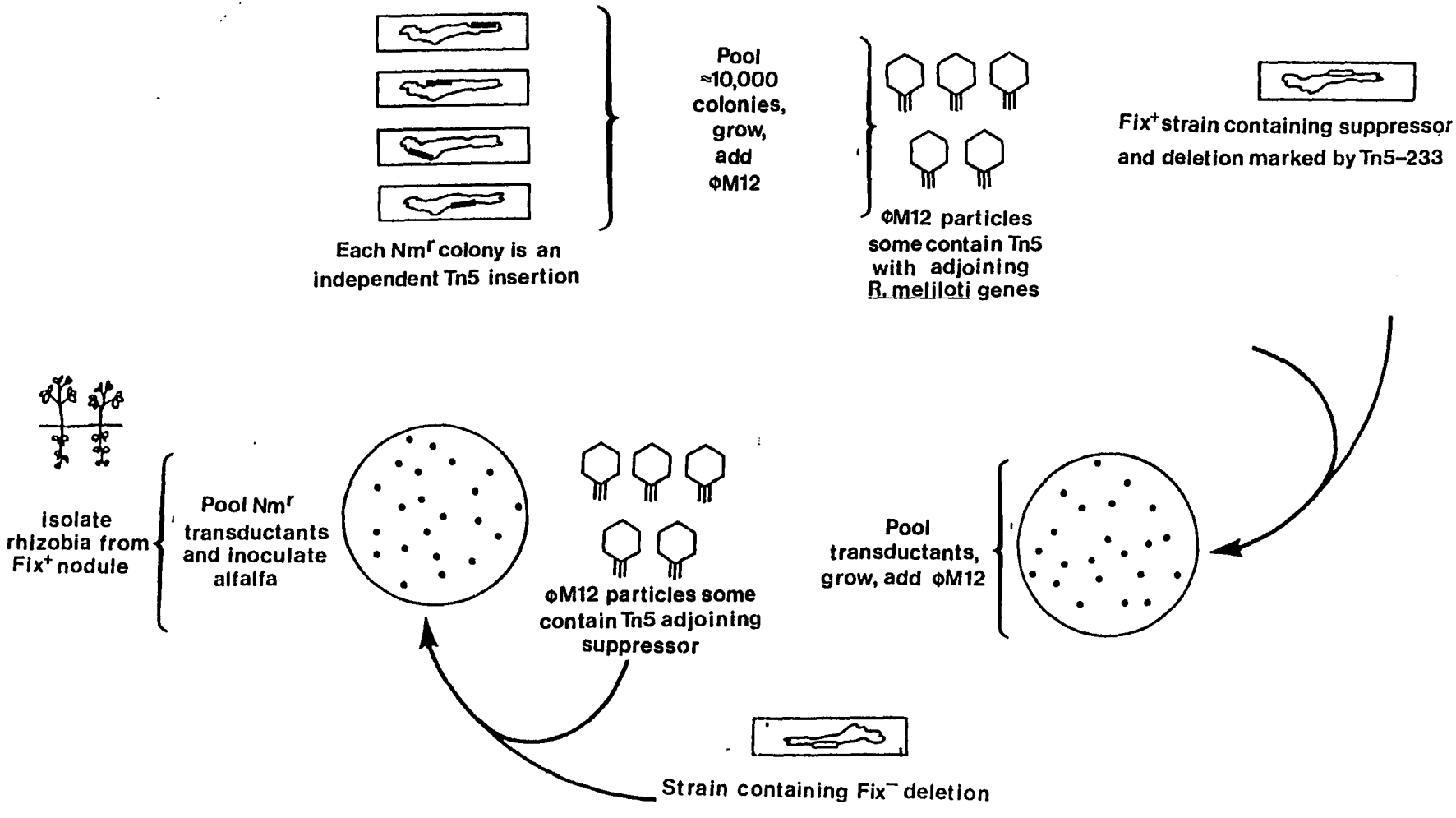
Initial work was carried out prior to the start of this M.Sc. project. To determine whether the second site mutation was linked to the Tn5-233 marking the deletion in RmF263 a lysate was grown on this strain and the Tn5-233 was transduced into a wild type Rm1021 background. Twenty Gm$^r$Sp$^r$ transductants were single colony purified three times and inoculated onto alfalfa. In each case the Tn5-233 cotransduced the Fix$^-$ deficiency. This suggested that the second site mutation was either unlinked or very loosely linked to Δ5408.

Isolation of Tn5 inserts linked to the sfx-1 mutation

Work done before the start of this project utilized the strategy shown in Figure 4 to isolate Tn5 inserts which were linked in transduction to the sfx-1 allele. Nm$^r$ was

Figure 4. Strategy for isolating Tn*5* insertion linked to *sfx-1*. A bank of random Tn*5* insertions in Rm1021 was transduced into a Fix$^+$ second site revertant to *fix-114*. The resultant transductants were pooled and a lysate was grown on these colonies and used to transduce a strain carrying a *fix-114* deletion to Fix$^+$. *Rhizobium* carrying a Tn*5* insertion linked to the second site mutation were isolated from pink Fix$^+$ nodules.

# STRATEGY FOR ISOLATING Tn5 INSERTIONS LINKED TO FIX SUPPRESSOR



transduced from 6,000 pooled random Tn5 inserts (Bank NM1) in Rm1021 into RmF263. The transductants were pooled, and a lysate was made. This lysate was then used to transduce $Nm^r$ into the $Fix^-$ strain Rm5408. Approximately 2,000 neomycin resistant transductant colonies were pooled and used to inoculate alfalfa. After three weeks it was observed that there was a mix of $Fix^+$ and $Fix^-$ nodules on the inoculated plants. Bacteria were isolated from the $Fix^+$ nodules and two independent isolates RmF338 and RmF339 were examined further. The Tn5 insertions in these strains were designated Ω5117 and Ω5118 respectively. Tn5-132 insertions were isolated which were linked to Ω5117 and Ω5118 in RmF338 and RmF339. Results of subsequent transductional crosses showed that both Ω5117 and Ω5118 were from the same region (Figure 5). Results of transductional crosses involving these and other markers suggest that Ω5117 and Ω5118 flank the sfx-1 allele (see below). Subsequent Tn5-233 inserts were also isolated and mapped to the sfx-1 region (Figure 6). Relevant inserts and cotransductional data of all relevant inserts is compiled in Figure 9.

## Mapping the sfx-1 allele by transduction

To map the sfx-1 allele in relation to the other inserts isolated a three factor cross was carried out. RmF379 carrying Ω5118::Tn5, $sfx\text{-}1^-$ in a Rm1021 background

FIGURE 5. TWO FACTOR COTRANSDUCTION FREQUENCIES OF Tn5-132 INSERTS LINKED TO Ω5117::Tn5 AND Ω5118::Tn5

A.

| Donor | Recepient | Marker selected | Recombinant classes | Linkage |
|---|---|---|---|---|
| (RmF378)->RmF424, | Ω5119::Tn5-132 | Nm | 46 $Ot^r$ 4 $Ot^s$ | 8% |
| (RmF379)->RmF424, | Ω5119::Tn5-132 | Nm | 9 $Ot^r$ 41 $Ot^s$ | 82% |
| (RmF378)->RmF425, | Ω5120::Tn5-132 | Nm | 49 $Ot^r$ 1 $Ot^s$ | 2% |
| (RmF379)->RmF425, | Ω5120::Tn5-132 | Nm | 2 $Ot^r$ 48 $Ot^s$ | 96% |
| (RmF378)->RmF426, | Ω5121::Tn5-132 | Nm | 44 $Ot^r$ 6 $Ot^s$ | 12% |
| (RmF379)->RmF426, | Ω5121::Tn5-132 | Nm | 16 $Ot^r$ 34 $Ot^s$ | 68% |
| (RmF378)->RmF427, | Ω5122::Tn5-132 | Nm | 40 $Ot^r$ 10 $Ot^s$ | 20% |
| (RmF379)->RmF427, | Ω5122::Tn5-132 | Nm | 34 $Ot^r$ 16 $Ot^s$ | 32% |
| (RmF378)->RmF428, | Ω5123::Tn5-132 | Nm | 36 $Ot^r$ 14 $Ot^s$ | 28% |
| (RmF379)->RmF428, | Ω5123::Tn5-132 | Nm | 34 $Ot^r$ 16 $Ot^s$ | 32% |
| (RmF378)->RmF429, | Ω5124::Tn5-132 | Nm | 49 $Ot^r$ 1 $Ot^s$ | 2% |
| (RmF379)->RmF429, | Ω5124::Tn5-132 | Nm | 26 $Ot^r$ 24 $Ot^s$ | 48% |
| (RmF378)->RmF431, | Ω5126::Tn5-132 | Nm | 27 $Ot^r$ 23 $Ot^s$ | 46% |
| (RmF379)->RmF431, | Ω5126::Tn5-132 | Nm | 50 $Ot^r$ 0 $Ot^s$ | 0% |
| (RmF378)->RmF994, | Ω5118::Tn5-233 | Nm | 91 $Gm^r$-$Sp^r$<br>6 $Gm^s$-$Sp^s$ | 6% |
| (RmF379)->RmF993, | Ω5117::Tn5-233 | Nm | 96 $Gm^r$-$Sp^r$<br>2 $Gm^s$-$Sp^s$ | 2% |

A. Lysates of strains RmF378 and RmF379 carrying Ω5117::Tn5 and Ω5118::Tn5 respectively were transduced into Tn5-132 insertions that were isolated as linked to either insert or into Tn5-233 replacements of Ω5117::Tn5 and Ω5118::Tn5 in strains RmF378 and RmF379 respectively. At least fifty neomycin transductants were screened in each case for loss of the appropriate marker. Frequencies are expressed as percent cotransduction.

B. A diagramatic representation of the data presented above. Note that the order of the Tn5-132 inserts relative to each other has not been established by three factor crosses and are therefore not absolute.

B

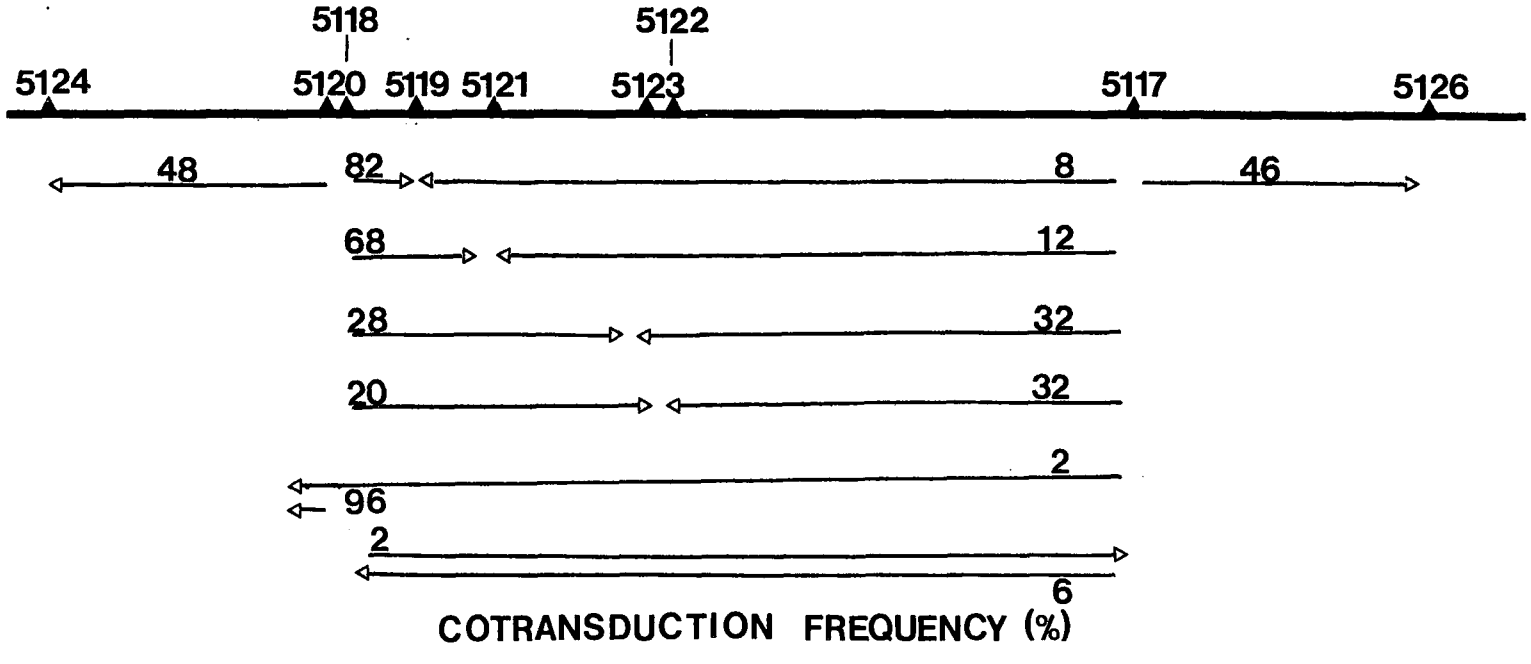
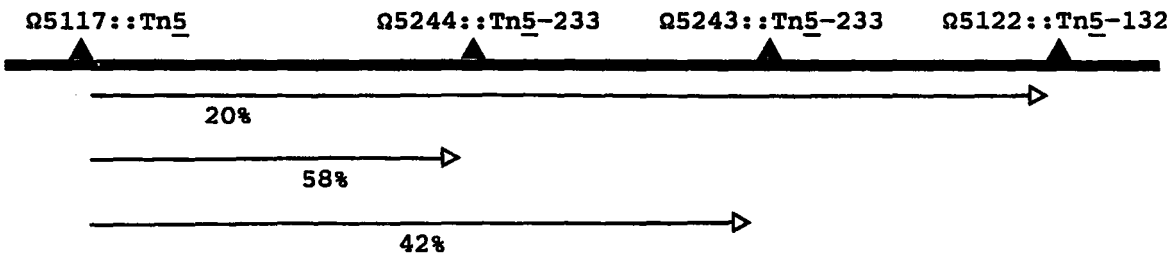

5118
5122
5124
5120
5119
5121
5123
5117
5126
48
82
8
46
68
12
28
32
20
32
2
96
2
6
COTRANSDUCTION FREQUENCY (%)

FIGURE 6. THREE FACTOR TRANSDUCTIONAL CROSS SHOWING POSITIONS OF Ω5243 AND Ω5244

| Donor Genotype | Recipient Genotype | Selected Phenotype | Class of Recombinant | No. in Class |
|---|---|---|---|---|
| Ω5117::Tn*5*, Ω5122::Tn*5*-132 | Ω5243::Tn*5*-233 | $Nm^r$ | $Gm^r$-$Sp^r$, $Ot^r$ | 0 |
| | | | $Gm^r$-$Sp^r$, $Ot^s$ | 28 |
| | | | $Gm^s$-$Sp^s$, $Ot^r$ | 10 |
| | | | $Gm^s$-$Sp^s$, $Ot^s$ | 11 |
| | Ω5244::Tn*5*-233 | $Nm^r$ | $Gm^r$-$Sp^r$, $Ot^r$ | 0 |
| | | | $Gm^r$-$Sp^r$, $Ot^s$ | 21 |
| | | | $Gm^s$-$Sp^s$, $Ot^r$ | 13 |
| | | | $Gm^s$-$Sp^s$, $Ot^s$ | 16 |

A lysate was made on RmG256 carrying Ω5117::Tn*5* and Ω5122::Tn*5*-132 and transduced into RmG351 and RmG352 containing Ω5243::Tn*5*-233 and Ω5244::Tn*5*-233 respectively. Fifty $Nm^r$ transductants were screened for cotransduction of $Ot^r$ and loss of $Gm^r$-$Sp^r$. The implied order is shown below.



was transduced into RmF427 carrying Ω5122::Tn*5*-132, *sfx-1*$^{+}$, Δ5408. Fifty neomycin resistant transductants were screened for loss of oxytetracycline and cotransduction of Fix$^{+}$. The results suggest that *sfx-1* maps beyond Ω5122 and is 20% linked to Ω5118 (Figure 7, see also Figure 9).

## Conjugal mapping of sfx-1 to the R. meliloti chromosome

The location of *sfx-1* on the chromosome was determined by mapping the *sfx-1* linked insertions Ω5117 and Ω5118. First the Nm$^{r}$ Tn*5* insertions, Ω5117 and Ω5118, in a Rm1021 background, were replaced with Tn*5*-233 (Gm$^{r}$,Sp$^{r}$) to give strains RmF993 and RmF994. Nm$^{r}$ from a series of Tn*5*-*mob* mapping strains was then transduced into RmF993 and RmF994. Thus a series of strains were constructed that contained Tn*5*-*mob* markers at different positions around the chromosome and either Ω5117::Tn*5*-233 or Ω5118::Tn*5*-233. These constructs were then mated with Rm5000 using pGMI102 as a mobilizing plasmid. Rf$^{r}$Gm$^{r}$Sp$^{r}$ transconjugants were scored and the results showed high conjugal linkage with strains transferring in a clockwise manner from *trp-33* (Table 3).

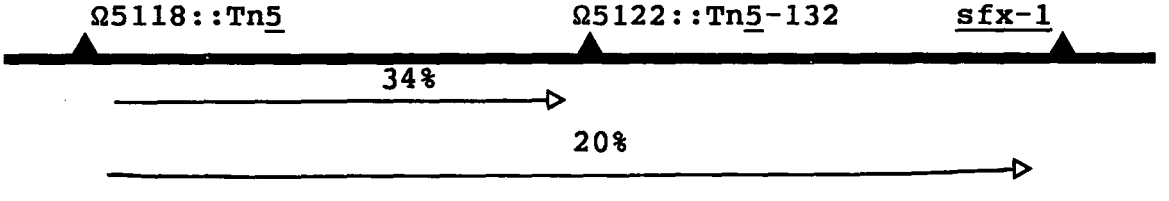
Figure 7

Three Factor Cross Showing Position of sfx-1

| Classes | # in class | Frequency (%) |
|---|---|---|
| $Ot^r$ $Fix^+$ | 0 | 0 |
| $Ot^r$ $Fix^-$ | 33 | 66 |
| $Ot^s$ $Fix^+$ | 10 | 20 |
| $Ot^s$ $Fix^-$ | 7 | 14 |

| | | Relevant Characteristics |
|---|---|---|
| Donor: | RmF379, Ω5118::Tn5, sfx-1$^-$ | $Ot^s$, $Nm^r$, $Fix^+$ |
| Recepient: | RmF427, Ω5122::Tn5-132 ΔΩ5033-Ω5064::Tn5-233, sfx-1$^+$ | $Ot^r$, $Nm^s$, $Fix^-$ |

Fifty $Nm^r$ transductants were screened for loss of Ot sensiitivity and Fix phenotype. Ot, oxytetracycline; Nm neomycin; sfx-1$^-$, suppressor allele (suppresses fix-114); sfx-1$^+$, wild type "suppressor locus" (does not suppress fix-114). The suggested order is shown below.



Other markers which map to the sfx-1 region

Other Tn5-oriT crosses that were done in the lab had suggested that pck-1 mapped in a counter clockwise orientation from Ω5122 (towards trp-33)(T. Finan, personal communication). It was found that Ω5117::Tn5-233 in RmF993 was not linked by transduction with pck-1::TnV (0/200 transductants screened). However Ω5118::Tn5-233 in RmF994 was weakly linked to pck-1::TnV (17/200 colonies screened had lost TnV::pck-1). This suggests that pck-1 is closely linked to trp-33 but the order about trp-33 was not determined.

The phe-54 marker was shown to be close and clockwise from trp-33 on the chromosomal map of Rm1021 (Meade and Signer 1977). phe-502 (unpublished) which maps to the same locus as phe-54 was transduced into Ω5117 and was found to be very tightly linked (94%). A subsequent three factor cross using RmG340 which carries insert Ω5232::Tn5-233 (designated as phe-232) as a recipient and RmG256 containing Ω5117::Tn5 and Ω5122::Tn5-132 as a donor lysate suggest that phe-232 lies between Ω5117 and Ω5122 (Figure 8).

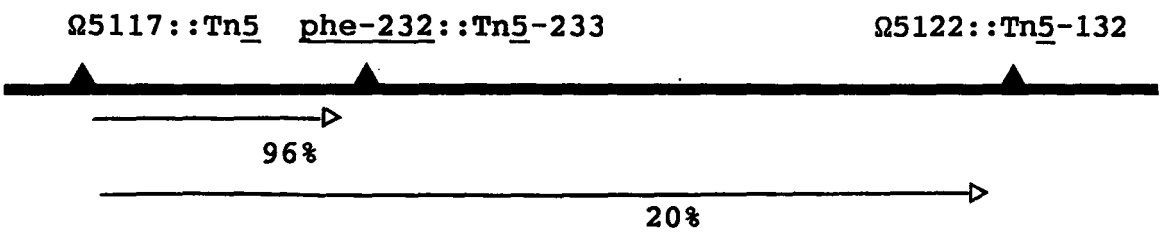
exoD17 maps in a clockwise orientation from trp-33 and is loosely linked in transduction to this marker (J. Glazebrook, personal communication). A three factor cross was done by transduction with a strain, RmG666, containing

Figure 8

Three Factor Cross Showing Position of phe-232

| Donor Genotype | Recipient Genotype | Class of Recombinant | No. in Class |
|---|---|---|---|
| Ω5117::Tn5<br>Ω5122::Tn5-132 | phe-232::Tn5-233 | $Gm^r$-$Sp^r$ $Ot^r$ | 0 |
| | | $Gm^r$-$Sp^r$ $Ot^s$ | 2 |
| | | $Gm^s$-$Sp^s$ $Ot^r$ | 8 |
| | | $Gm^s$-$Sp^s$ $Ot^s$ | 40 |

A lysate was made on a strain carrying Ω5117::Tn5 and Ω5122::Tn5-132 and transduced into strain containing phe-232::Tn5-233. Fifty $Nm^r$ transductants were screened for cotransduction of $Ot^r$ and loss of $Gm^r$-$Sp^r$. The implied order is shown below.



Ω5118::Tn*5*, Ω5122::Tn*5*-132 as a donor, and RmG590 containing *exoD17*::Tn*5*-233 as a recipient. The results imply that *exoD17* is 6% linked in transduction to Ω5118::Tn*5* (Figure 9). This result shows that *sfx-1* does not appear to be *exoD* since they appear to map to different positions in Rm1021. The results, however, should be viewed with caution since repeated crosses using these strains continually yielded low numbers of transductants. Since *exoD17* is involved the production of succinoglycan, an outer membrane component, it may be that the resulting combination of mutations produced by this cross may be creating a severely compromised bacterial strain which may not have a high survival rate and thus skew the transductional frequencies. The implied map order, however, does exclude the possibility that *sfx-1* is a mutation at the *exoD* locus.

All relevant markers and inserts in the *trp-33* *sfx-1* region have been included in Figure 10.

## Cloning of the sfx-1 locus

Two independent cosmid banks were constructed with partial *Bam*H1 digested DNA from RmF263 (carrying *sfx-1* allele). The vector used was the 12 kb $Tc^r$ cosmid pRK7813 (Jones and Gutterson 1987). This vector is a RK2 derivative and is mobilizable by pRK2013 or pRK600. The banks consisted of 2400 and 2250 pooled colonies respectively.

FIGURE 10. THREE FACTOR CROSS SHOWING POSITION OF exoD17

| Donor Genotype | Recipient Genotype | Selected Genotype | Class of Recombinant | No. in Class |
|---|---|---|---|---|
| Ω5118::Tn5, Ω5122::Tn5-132 | exoD17::Tn5-233 | $Nm^r$ | $Ot^r$ $Gm^r$-$Sp^r$ | 14 |
| | | | $Ot^r$ $Gm^s$-$Sp^s$ | 1 |
| | | | $Ot^s$ $Gm^r$-$Sp^r$ | 71 |
| | | | $Ot^s$ $Gm^s$-$Sp^s$ | 4 |

A lysate of RmG666, containig Ω5118::Tn5 and Ω5122::Tn5-132, was transduced into RmG590 containing exoD17::Tn5-233. Ninety $Nm^r$ transductants were screened for cotransduction of $Ot^r$ and for loss of $Gm^r$-$Sp^r$. The implied order is shown below.

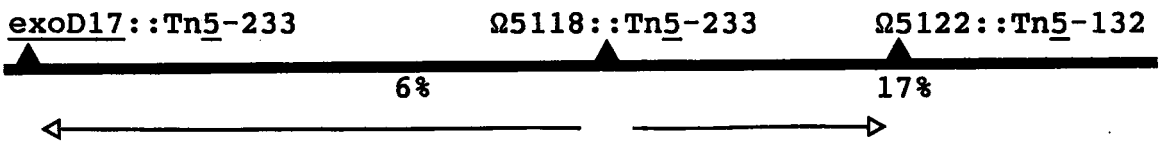
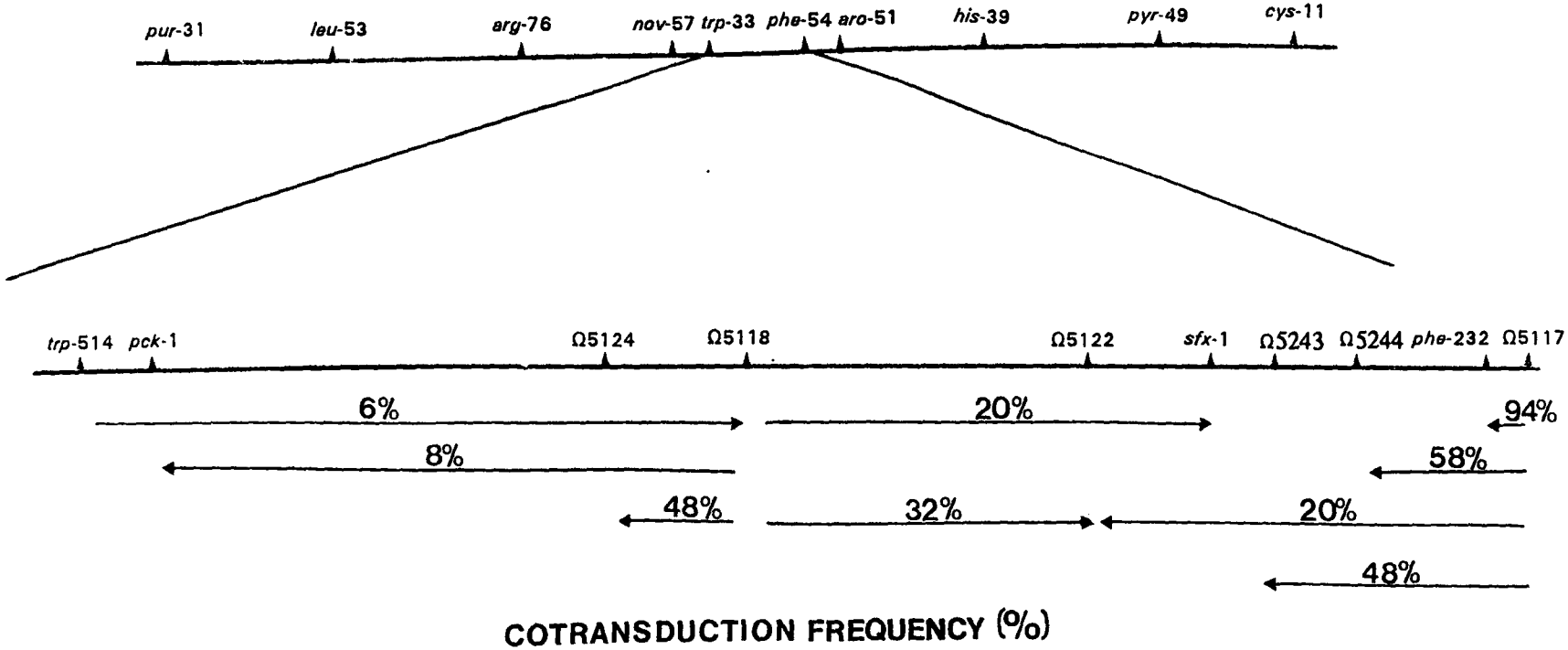


Figure 10. Compilation of all relevant markers in the sfx-1 region in relation to Meade and Signers (1977) chromosomal map of Rhizobium meliloti. Clockwise transfer is equivalent to moving from left to right on this linear representation.


pur-31
leu-53
arg-76
nov-57
trp-33
phe-54
aro-51
his-39
pyr-49
cys-11
trp-514
pck-1
Ω5124
Ω5118
Ω5122
sfx-1
Ω5243
Ω5244
phe-232
Ω5117
6%
20%
94%
8%
58%
48%
32%
20%
48%
COTRANSDUCTION FREQUENCY (%)

This corresponds to a packaging efficiency of 6.08 X $10^3$ cfu/$\mu$g of fractionated DNA at a ratio 10:1 (insert:vector), 2.04 X $10^3$ cfu/$\mu$g fractionated DNA for a 5:1 ratio, 1.6 X $10^3$ cfu/$\mu$g fractionated DNA for a 2:1 ratio, and 2.8 X $10^3$ cfu/$\mu$g fractionated DNA at a 1:2 ratio. The cosmid banks were mated into RmF114 and Rm5408 and between 14,000 and 31,000 $Sm^rTc^r$ transconjugants were pooled and inoculated onto alfalfa seedlings planted in Leonard pot assemblies.

After twenty days, plants that had been inoculated with RmF114 or Rm5408 with the clone bank present definitely looked like they were fixing nitrogen. Nodules from these plants were surface sterilized either individually or in a group of ten and spread on LB Tc (10 $\mu$gm/ml) agar. Resultant colonies were purified once and plasmids were mated into E. coli MT607. Restriction analysis of 36 isolates yielded three distinct cosmid types. Representatives of two classes were designated pTH56 and pTH57. The third cosmid class did not contain any insert. This latter type presumably represents a packaged vector concatamer that was mated into a cell in which a suppressor arose.

Restriction analysis of plasmids pTH56 and pTH57 revealed each contained an 18 kb BamH1 fragment in common. Gel isolation, and labelling of the 18 kb BamH1 fragment from pTH56 showed that this hybridized to the 18 kb fragment

from pTH57 confirming that these two cosmids carried a common BamH1 fragment (Figure 11).

pTH56 contains DNA that is from the sfx-1 region

To confirm that pTH56 contained DNA from the sfx-1 region Southern Blot analysis was carried out. Tn5-233 insertions Ω5243 and Ω5244 in strains RmG351 and RmG352 respectively had been mapped by three factor cross to lie near sfx-1 between Ω5117 and Ω5122 (Figure 6). Total DNA was isolated from these strains, cut with BamH1, EcoR1, HindIII, and probed with digoxigenin labelled pTH56 (Figure 12). The results show that Ω5243 lies within the 18 kb BamH1 fragment (lane c) of pTH56 and Ω5244 is within the 7 kb BamH1 fragment of pTH56 (lane b). This confirms that insert DNA in pTH56 is contiguous with the sfx-1 region that had been mapped genetically. Strains RmG338 containing Ω5230::Tn5-233, RmG167 containing Ω5122::Tn5-132 and RmF428 containing Ω5123::Tn5-132 were also probed with labellled pTH56 and pTH57 and did not show any shift in the expected hybridization pattern.

Figure 11. A) TAE agarose gel electrophoresis of restricted pTH56 and pTH57. Lane 1, pTH57 HindIII digest; Lane 2, pTH56 HindIII digest; Lane 3, pTH57 BamHI,HindII double digest; Lane 4, pTH56 BamHI HindIII double digest; Lane 5, pTH57 BamHI digest: Lane 6, pTH56 BamHI digest.

B) Southern analysis of pTH56 and pTH57. The 18 kb BamHI fragment from pTH56 was isolated from a low melting point gel, labelled and used to probe restricted pTH56 and pTH57. Lanes are as indicated in part A.

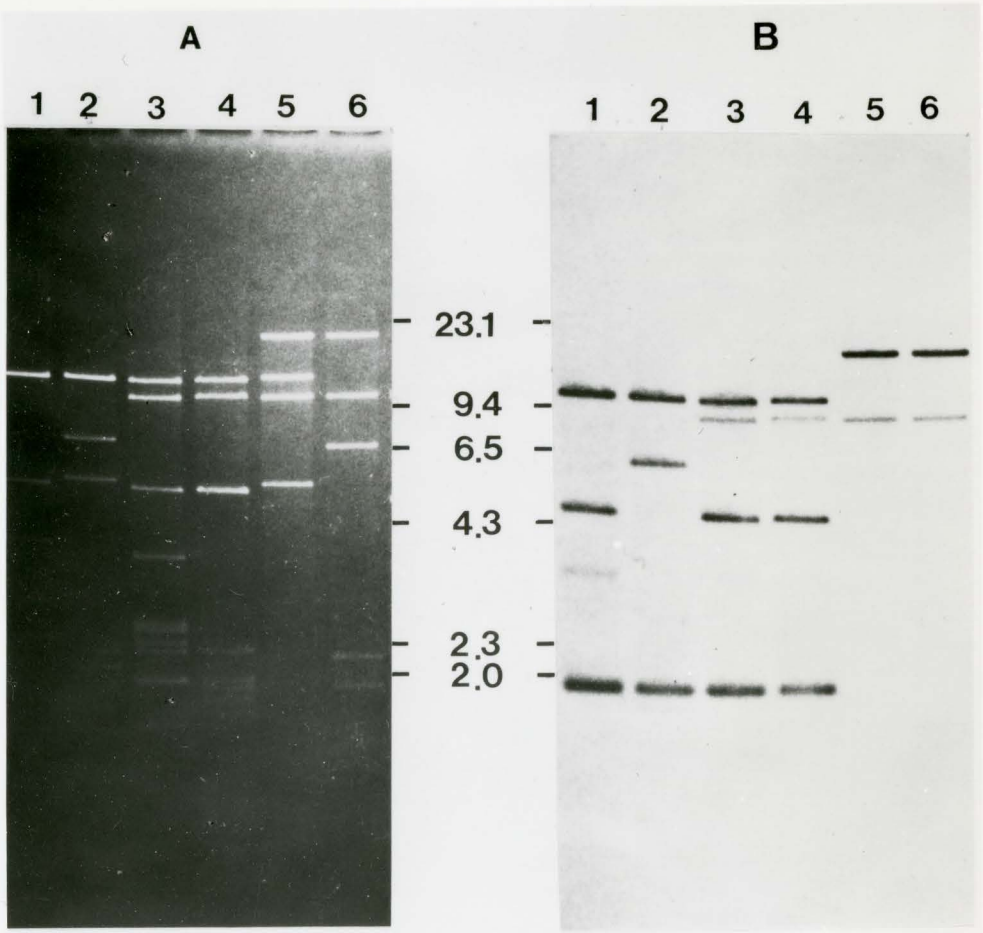
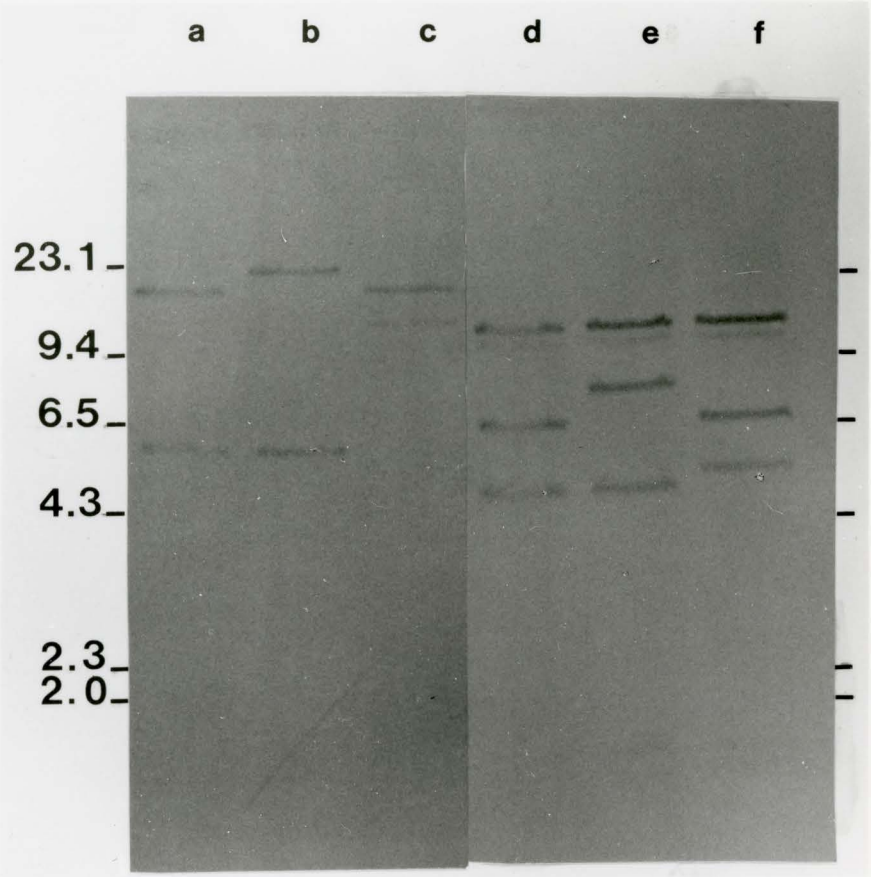

A
B
1 2 3 4 5 6
1 2 3 4 5 6
23.1
9.4
6.5
4.3
2.3
2.0

Figure 12. Southern analysis of RmG350 and RmG351. Total DNA from Rm1021, RmG350 carrying Ω5243::Tn*5*-233, and RmG351 carrying Ω5244::Tn*5*-233 was isolated and restricted with *Bam*HI (lanes a, b, and c) or with *Hin*dIII (lanes d, e, and f) and probed with labelled pTH56. In each case two smaller bands around 2 kb did not resolve. Lane a, Rm1021; Lane b, RmG350, Lane c, RmG351; Lane d, Rm1021; Lane e, RmG350; Lane f, RmG351.


a
b
c
d
e
f
23.1
9.4
6.5
4.3
2.3
2.0

## pTH56 suppresses the Fix- phenotype of ΔF114, Δ5408 and the osmotic phenotype of Fix114 mutations

Plasmids pTH56 and pTH57 were transferred from *E. coli* into Rm5408 and RmF114 and the resulting transconjugants were tested on alfalfa for restoration of the symbiotic phenotype. Three independent cultures of RmF114 or Rm5408 with either pTH56 or pTH57 were inoculated onto five day old alfalfa seedlings. After 23 days the root nodules were assayed by the acetylene reduction assay for nitrogenase activity and the shoots of the plants were used to assay cumulative nitrogen fixation (Table 4). The results indicate that the cosmids restore a $Fix^+$ phenotype to the $Fix^-$ deletion strains. The suppression, however, was variable. This may be due to either plasmid instability, plasmid copy number, or perhaps to a co-dominant effect between the suppressor and the wild type allele.

pTH56 was also mated into RmG439, which contains a 8 kb deletion of *fix114*, and into single Tn*5* insertion mutants in *fix114* and screened for suppression of the mucoid phenotype associated with *fix114*. The results showed that pTH56 suppressed the mucoid phenotype of the deletion as well as all Tn*5* inserts in *fix114* (Table 5).

TABLE 4. SUPPRESSION OF Rm5408 AND RmF114 ALFALFA FIX DEFICIENCY BY pTH56 AND pTH57

| STRAIN | ARA[a] nM/plant/h ± SE[b] | % W.T. ARA | Dry Wt mg/plant[c] | % W.T. Dry Wt |
|---|---|---|---|---|
| Rm1021 | 818.7 ± 22.2 | 100 | 15.4 | 100 |
| RmF114 | 29.3 ± 3.3 | 4 | 5.9 | 38 |
| Rm5408 | 97.0 ± 7.7 | 12 | 6.4 | 42 |
| Rm5408(pTH56) | 994.7 ± 6.1 | 121 | 14.4 | 94 |
| Rm5408(pTH57) | 595.8 ± 68.6 | 73 | 11.1 | 72 |
| RmF114(pTH56) | 622.4 ± 43.5 | 76 | 10.5 | 68 |
| RmF114(pTH57) | 329.7 ± 51.7 | 40 | 10.2 | 66 |
| RmF263 | 952.0 ± 66.7 | 116 | 17.7 | 115 |
| Uninoculated | 16.0 ± 0.0 | 2 | 5.7 | 37 |

[a] Acetylene Reduction Activity

[b] In all cases the root systems of at least nine plants were assayed.

[c] For dry weight analysis the shoots of at least 26 plants were weighed.

TABLE 5. PHENOTYPE OF *fix114* MUTANTS ON LOW OSMOLARITY MEDIA IN THE PRESENCE AND ABSENCE OF PLASMID pTH56

| Strains | Relevant Characteristics | 1/2 GYM | 1/2 GYM (100 mM NaCl) |
|---|---|---|---|
| Rm1021 | Wild type | - | - |
| RmG439 | 8 kb deletion of *fix114* | + | - |
| RmG439(pTH56) | | - | - |
| RmF919 | *fix114-1*::Tn*5* | + | - |
| RmF919(pTH56) | | - | - |
| RmF921 | *fix114-4*::Tn*5* | + | - |
| RmF921(pTH56) | | - | - |
| RmF922 | *fix114-5*::Tn*5* | + | - |
| RmF922(pTH56) | | - | - |

pTH56, carrying *sfx-1*, was mated into each of the strains and two transconjugants from each were tested on low osmolarity media. + = mucoid colony morphology, - = dry colony morphology.

## TnphoA mutagenesis of pTH56

TnphoA mutagenesis of fix114 yielded active fusions which were Fix⁻ when homogenotized into a wildtype background and inoculated onto alfalfa (T. Charles, Ph.D thesis). It was considered likely that sfx-1 may also encode a membrane protein. pTH56 was therefore mutagenized with TnphoA.

pTH56 was passaged through E. coli MT621, which contains TnphoA in the chromosome, and mated into RmF222 and colonies that were blue on LB plates containing X-phos were isolated. Seven plasmids carrying independent active TnphoA insertions were isolated and mated into MT607 for restriction analysis. On the basis of BamH1 and HindIII digests, six insertions (Ω9B, Ω6, Ω20B, Ω13, Ω21A, Ω21B) were localized within the 14 kb HindIII fragment that was internal to the 18 kb BamH1 fragment and a single insertion, Ω9A, was mapped to a 5.7 kb HindIII fragment of pTH56. This latter fragment is not common to pTH57 (Figure 11 a).

To determine if any of the TnphoA inserts were within the sfx-1 gene the plasmids with the TnphoA inserts were introduced into RmF114, Rm5408, and RmG439. Rm5408 and RmF114 with fusion plasmids were then tested for their ability to suppress the Fix⁻ phenotype on alfalfa. Plasmids

introduced into RmG439 were tested for there ability to suppress the mucoid phenotype of fix114 on low osmolarity media. Dry weight analysis (Table 6) and low osmolarity tests (Table 7) both are consistent with the conclusion that none of these inserts were in the sfx-1 gene.

Interestingly when TnphoA insertions were recombined into RmG591 which contains the sfx-1 allele, strains containing inserts Ω9, Ω13, or Ω20B were sensitive to the detergents deoxycholate, SDS, sarkosyl, and to the antibiotic bacitracin (Table 8). Sensitivity to these compounds is usually associated with alterations in the outer membrane of other Gram negative bacteria (Nikaido et al. 1985). These homogenotes were confirmed by Southern blot analysis and one insert, Ω21A::TnphoA in strain RmG628, was mapped by a three factor transductional crosses (Figure 13). Four of the homogenotes, RmG624, RmG626, RmG627, and RmG628 carrying Ω9A::TnphoA, Ω13::TnphoA, Ω20B::TnphoA, and Ω21A::TnphoA respectively were also inoculated onto alfalfa to test for symbiotic phenotypes. One strain, RmG626, carrying Ω13::phoA had a Fix$^-$ phenotype (Appendix 2).

## Tn5 mutagenesis of pTH56

To isolate a Tn5 insertion in the sfx-1 locus pTH56 was mutagenized with Tn5 and mated into RmG490. Four colonies from each of 10 independent matings were screened on low

TABLE 6. SYMBIOTIC PHENOTYPE OF Rm5408 AND RmF114 CONTAINING pTH56 WITH ACTIVE TnphoA INSERTIONS

| Strain | No. of plants | Shoot dry weight mg/plant ± SE | % Wild Type |
|---|---|---|---|
| Rm1021 | 18 | 46.25 ± 6.4 | 100 |
| Uninoculated | 18 | 3.85 ± 0.7 | 8 |
| Rm5408 | 20 | 5.15 ± 0.2 | 11 |
| RmF114 | 18 | 5.05 ± 0.9 | 11 |
| RmF263 | 15 | 58.80 ± 8.3 | 127 |
| Rm5408/pTH56 | 16 | 36.65 ± 8.9 | 79 |
| RmF114/pTH56 | 19 | 27.50 ± 6.8 | 59 |
| Rm5408/pTH56Ω6 | 17 | 36.25 ± 3.35 | 78 |
| RmF114/pTH56Ω6 | 18 | 25.90 ± 3.5 | 56 |
| Rm5408/pTH56Ω9A | 17 | 33.25 ± 4.1 | 72 |
| RmF114/pTH56Ω9A | 20 | 40.65 ± 10.4 | 88 |
| Rm5408/pTH56Ω13 | 18 | 33.40 ± 0.4 | 72 |
| RmF114/pTH56Ω13 | 16 | 22.70 ± 3.1 | 49 |
| Rm5408/pTH56Ω20B | 16 | 35.45 ± 4.3 | 77 |
| RmF114/pTH56Ω20B | 15 | 22.00 ± 2.1 | 48 |
| Rm5408/pTH56Ω21A | 18 | 30.75 ± 1.2 | 66 |
| RmF114/pTH56Ω21A | 17 | 41.15 ± 11.2 | 90 |
| Rm5408/pTH56Ω21B | 17 | 35.25 ± 7.1 | 76 |
| RmF114/pTH56Ω21B | 18 | 29.75 ± 0.1 | 64 |

pTH56 containing active TnphoA fusions were mated into either Rm5408 or RmF114 and these constructs were then inoculated onto alfalfa. Plants were 28 days old when they were harvested. Values are presented as the mean ± standard error of 3 replicates.

TABLE 7. EFFECT OF TnphoA INSERTS IN pTH56 ON OSMOLARITY PHENOTYPE OF RmG439

| Strain | 1/2 GYM (colony phenotype) |
|---|---|
| Rm1021 | dry |
| RmG439 | mucoid |
| RmG439/pTH56 Ω6 | dry |
| RmG439/pTH56 Ω9A | dry |
| RmG439/pTH56 Ω9B | dry |
| RmG439/pTH56 Ω13 | dry |
| RmG439/pTH56 Ω20B | dry |
| RmG439/pTH56 Ω21A | dry |
| RmG439/pTH56 Ω21B | dry |
| RmG439/pTH56 | dry |

pTH56 containing TnphoA were mated into RmG439 and single transconjugants were tested on 1/2 GYM and 1/2 GYM supplemented with 100 mM NaCl. All strains were streaked onto 1/2 GYM supplemented with 100 mM NaCl had a dry colony morphology. Plates were scored after 5 days at 30°.

TABLE 8. PHENOTYPE OF TnphoA HOMOGENOTES IN sfx-1 REGION

| Strains | Suc | Glu | Doc | SDS | Sark | Bac | Calc |
|---|---|---|---|---|---|---|---|
| Rm1021 wild-type | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| RmF379 sfx-1 | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| RmG623 Ω6::TnphoA | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| RmG624 Ω9A::TnphoA | ++ | ++ | +/- | ++ | ++ | + | +[a] |
| RmG625 Ω9B::TnphoA | ++ | ++ | - | - | +/- | ++ | ++ |
| RmG626 Ω13::TnphoA | ++ | ++ | - | - | +/- | +/- | +[a] |
| RmG627 Ω20B::TnphoA | ++ | ++ | - | - | - | + | +[a] |
| RmG628 Ω21A::TnphoA | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| RmG629 Ω21B::TnphoA | ++ | ++ | ++ | ++ | ++ | ++ | ++ |

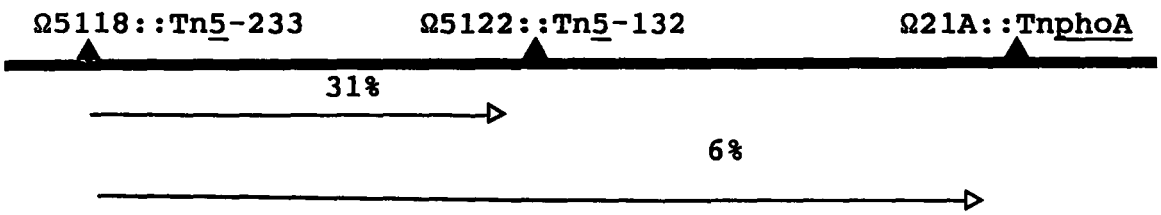
Abbreviations: Growth of M9 agar containing 15 mM succinate (Suc), 15 mM glucose (Glu)and on LB agar supplemented with 2 mg/ml desoxycholate (Doc), 0.1 mg/ml SDS; 1 mg/ml sarkosyl (Sark); 0.1 mg/ml bacitracin (Bac); and with 0.2% calcofluor (Calc). ++, good growth; +, poor growth small colonies; +/-, no single isolated colonies; -, no growth.

[a] Growth by these strains on media containing calcofluor was very poor but colonies appeared bright under UV light.

FIGURE 13. THREE FACTOR CROSS SHOWING POSITION OF A TnphoA FUSION ISOLATED FROM pTH56

| Donor Genotype | Recipient Genotype | Selected Phenotype | Class of Recombinant | No.in Class |
|---|---|---|---|---|
| Ω5118::Tn5-233, Ω5122::Tn5-132 | Ω21A::TnphoA | $Gm^r$-$Sp^r$ | $Nm^r$ $Ot^r$ | 25 |
| | | | $Nm^r$ $Ot^s$ | 69 |
| | | | $Nm^s$ $Ot^r$ | 6 |
| | | | $Nm^s$ $Ot^s$ | 0 |

A lysate was made on strain RmG667, containing Ω5118::Tn5-233 and Ω5122::Tn5-132, and transduced into RmG628, which contains Ω21A::TnphoA which was isolated from pTH56 and subsequently homogenotized. One hundred $Gm^r$-$Sp^r$ colonies were screened for cotransduction of $Ot^r$ and loss of $Nm^r$. The implied order is shown below.



osmolarity media for loss of suppression of the mucoid phenotype associated with fix114. Of forty colonies screened, one insert, designated as 10A, fell into this category. E. coli strains carrying these plasmids were frozen for analysis in the future.

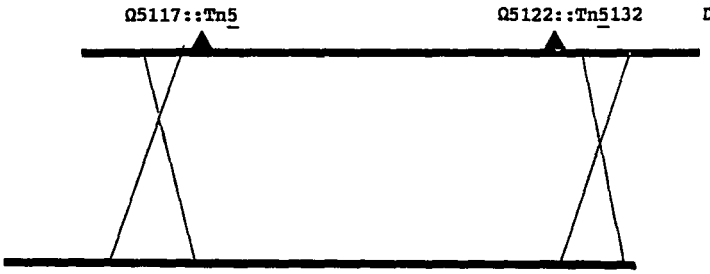
## CHAPTER 5

## SECOND SITE SUPPRESSORS TO FIX114 FALL INTO TWO CLASSES

### sfx-2 and sfx-3 do not map to the sfx-1 region

To determine if all of the five second site fix114 suppressor mutations mapped to the sfx-1 region, strains were constructed by transducing RmF378 containing Ω5117::Tn5 (but not sfx-1) into RmG167 which contains Ω5122::Tn5-132 and screening the resultant colonies for both markers. Together these insertions flank the sfx-1 locus (Figure 14). Four such transductants were single colony purified three times and were designated RmG256, RmG257, RmG258, and RmG259. Lysates were grown on these strains and each of these was used to transduce $Nm^r$ into the four independent $Fix^+$ pseudorevertant strains RmF263, RmF346, RmG203, and RmG204 carrying sfx-1, sfx-2, sfx-4, and sfx-5 (Note that RmG425 which was subsequently designated as carrying sfx-3 was not isolated at the time of this experiment). Two $Nm^r$, $Ot^r$ colonies from each of the four independent crosses were purified and examined for their symbiotic phenotype on alfalfa (Figure 14).

FIGURE 14. LOCALIZATION OF CLASS I MUTATIONS




Donor: Φ(RmG256), carrying wild type DNA between Ω5117 and Ω5122

Recipient: Fix$^+$ pseudorevertant carrying Fix deletion (Δ5408 or ΔF114)

Two Nm$^r$, Ot$^r$ recombinants from each of 4 independent crosses were inoculated onto alfafa

| Strain | Genotype | Nm$^r$ | Ot$^r$ | Fix |
|---|---|---|---|---|
| Rm1021 | wild type | - | - | + |
| Recombinants | | + | + | + |
| Rm5408 | Δ5408 | - | - | - |
| RmF114 | ΔF114 | - | - | - |
| RmF263 | Δ5408, *sfx-1* | - | - | + |
| Recombinants | | + | + | - |
| RmG203 | Δ5408, *sfx-4* | - | - | + |
| Recombinants | | + | + | - |
| RmG204 | ΔF114, *sfx-5* | - | - | + |
| Recombinants | | + | + | - |
| RmF346 | ΔF114, *sfx-2* | - | - | + |
| Recombinants | | + | + | + |

RmG256, RmG257, RmG258, and RmG259 were constructed by transduction such that Ω5117::Tn5 and Ω5122::Tn5-132 would flank the *sfx-1+* locus. Lysates were grown on these strains and Nm$^r$ was transduced into each of the *fix-114* second site revertants. Two Nm$^r$ Ot$^r$ transductants from each cross were purified and inoculated onto alfalfa.

The results showed that $Nm^r$ $Ot^r$ recombinants of RmF263, RmG203, and RmG204 formed $Fix^-$ nodules when inoculated on alfalfa. Thus the second site fix114 suppressor mutations in these strains (sfx-1, sfx-4, and sfx-5) lie within or are tightly linked to the region flanked by the inserts Ω5122 and Ω5117. In contrast none of the eight $Nm^r$ $Ot^r$ recombinants of strain RmF346 formed $Fix^-$ nodules. The second site fix114 suppressor mutation, sfx-2, in RmF346 is not tightly linked to the Ω5122-Ω5117 region (Figure 14).

## Strains carrying Class II, but not Class I second site revertants are sensitive to detergents

In an effort to better characterize the second site revertant strains they were tested for growth and sensitivity to a number of phage and chemical compounds. It was found that neither deletion (ΔF114 or Δ5408) nor the fix114 second site revertants showed any phage phenotype (Table 9). When the mutants were tested on a variety of detergent media it was found that RmF346 carrying sfx-2 and RmG425 carrying sfx-3 were sensitive to deoxycholate, SDS, and sarkosyl and to the antibiotic bacitracin (Table 9). On the basis of the detergent phenotype (Table 9), the genetic data (Figure 14), and the transductional linkage of sfx-2 and sfx-3 (Table 10, see below), the pseudorevertants were divided into two classes; Class I

TABLE 9. PHENOTYPES OF SECOND SITE REVERTANTS TO *fix114*

| Strains | Glu | Doc | Sark | SDS | Bac | Phage[a] |
|---|---|---|---|---|---|---|
| Rm1021 (Wildtype) | ++ | ++ | ++ | ++ | ++ | ++ |
| RmF263 (Class I) | ++ | ++ | ++ | ++ | ++ | ++ |
| RmF346 (Class II) | ++ | +/- | +/- | +/- | +/- | ++ |
| RmF114 (ΔF114) | ++ | ++ | ++ | ++ | ++ | ++ |

Abbreviations: Glu, M9 agar supplemented with 15 mM glucose; Doc, LB agar supplemented with 2 mg/ml deoxycholate; Sark, LB agar supplemented with 1 mg/ml sarkosyl; SDS, LB agar supplemented with 0.1 mg/ml SDS; Bac, LB agar supplemented with 0.1 mg/ml Bacitracin; Phage, sensitivity to a number of *R. meliloti* phage (see below); ++, good growth (like wildtype); +, poor growth (tiny colonies); +/-, poor growth (no single colonies); -, no growth.

[a] Phage tested include; ΦM1, ΦM5, ΦM7, ΦM9, ΦM10, ΦM11, ΦM12, and ΦM14. All strains formed turbid spots with ΦM7, clear spots were formed by all other phage tested.

consisting of RmF263, RmG203, and RmG204 carrying sfx-1, sfx-4, and sfx-5 respectively , and Class II consisting of RmF346 and RmG425 carrying sfx-2, and sfx-3 respectively.

## Isolation of Tn5 inserts linked to sfx-2

In order to further manipulate and to map the sfx-2 locus a Tn5 insert linked in transduction to the sfx-2 locus was isolated by transducing a lysate grown on 6,000 random Tn5 insertions (Bank NM1) into RmF346 containing the sfx-2 allele and screening individual $Nm^r$ transductants for loss of sensitivity on deoxycholate/EDTA media. Six Tn5 insertions Ω5254, Ω5255, Ω5256, Ω5257, Ω5258, and Ω5259, in strains RmG549, RmG550, RmG551, RmG552, RmG655, and RmG656 respectively were isolated as linked in transduction to deoxycholate sensitivity in RmF346 with linkages ranging from 4% to 70%. One insert, Ω5258::Tn5, which was 70% linked to deoxycholate sensitivity was transduced into Rm1021 and designated RmG558. To isolate Tn5-233 insertions, linked in transduction to the sfx-2 region, a bank, Bank GS2, consisting of approximately 2000 pooled Tn5-233 insertions was transduced into RmG558 containing Ω5258::Tn5. Three hundred transductants were screened and three colonies were isolated as $Nm^s$ $Gm^rSp^r$. They were subsequently found to be 26%, 60%, and 28% linked to Ω5258::Tn5 respectively, and were designated RmG639 carrying

Ω5256::Tn*5*-233, RmG640 carrying Ω5263::Tn*5*-233, and RmG641 carrying Ω5264::Tn*5*-233.

## *sfx*-2 and *sfx*-3 map to the same locus

Insertion Ω5258::Tn*5* was isolated as 70% linked to the deoxycholate sensitivity in RmF346 (see above). To determine whether the *sfx*-3 mutation in RmG425 was also linked to this insert a lysate was grown on a wild type strain RmG551 containing Ω5258::Tn*5* and $Nm^r$ was transduced into the other Class II revertant, RmG425, containing *sfx*-3. The results showed that Ω5258 was about 70% linked to deoxycholate sensitivity in strain RmF346 and RmG425 suggesting that *sfx*-2 and *sfx*-3 map to the same locus (Table 10). This, however, was not confirmed by a three factor cross.

## Inserts linked to class II revertants map to the chromosome

To determine whether class II mutations mapped to the chromosome of Rm1021 a series of Tn*5*-*mob* markers able to mobilize the entire chromosome in segments (Klein 1987) was introduced into RmG640 (see above). These constructs were mated with Rm5000 and *E. coli* MM294A containing pGMI102 and $Rf^r$,$Gm^rSp^r$ were scored as number of recombinants per $10^8$

TABLE 10. LINKAGE OF *sfx-2* AND *sfx-3* TO Ω5258

| Donor lysate | Recipient strain | Recombinant Class | % Linkage |
|---|---|---|---|
| RmG551 *sfx-2*$^{+}$, Ω5258::Tn*5*, | RmF346 *sfx-2* | 70 DE$^{r}$, 30 DE$^{s}$ | 70 |
| | RmG425 *sfx-3* | 72 DE$^{r}$, 28 DE$^{s}$ | 72 |

A lysate of RmG551 containing Ω5258::Tn*5* 70% linked to the deoxycholate sensitivity in RmF346 was transduced into both RmF346 and RmG425. Nm$^{r}$ colonies were screened for sensitivity on LB containing Deoxycholate (1mg/ml) and EDTA (0.25 mM).

TABLE 11. CONJUGAL MAPPING OF Ω5263, AN INSERT 70% LINKED IN TRANSDUCTION TO Ω5258

| Donor strain[a] | Number of Recombinants / $10^8$ cell[b] |
|---|---|
| leu-53 (+) | 0[c] |
| leu-53 (-) | 0 |
| trp-33 (+) | 2.8 X $10^3$ |
| trp-33 (-) | 0 |
| pyr-49 (+) | 0 |
| pyr-49 (-) | 0 |

[a] Donor strains contained Ω5263::Tn5-233 and the Tn5-mob markers indicated. Tn5-mob markers are linked in transduction to the auxotrophic markers indicated and transfer in either a clockwise (+) or counterclockwise (-) direction.

[b] Equal volumes of donor and recipient were mated together using MM294A(pGMI102) as a mobilizing strain and Rm5000 as a recipient strain (see methods). $Rf^r$ Gm-$Sp^r$ recombinants were selected and expressed as cfu/$10^8$ donor cells.

[c] No recombinants detected.

donor cells. The results suggest that Ω5263::Tn*5*-233 maps in a clockwise orientation from the chromosomal *trp-33* marker on Meade and Signer's (1977) map of Rm1021 (Table 11).

## pTH23 reverses the deoxycholate sensitive phenotype of Class II second site revertants

RmG479 carrying *sfx-2* in a wild type background was made by T. Charles by transducing Ω5025::Tn*5*, which is internal to ΔF114, from Rm5348 into RmF346 and thereby adding back the DNA which was deleted in ΔF114. The resultant strain, RmG479, did not show any deoxycholate sensitive phenotype. The sensitivity to deoxycholate was subsequently found to be dependent upon a region within ΔF114 and more specifically in the area that is responsible for the exopolysaccharide deficiency (calcofluor dark) phenotype (T. Charles, personal communication). Cosmid pTH23, which complements the dark phenotype of ΔF114 but not the Fix$^-$ was mated into both of the Class II second site revertants. The results showed that pTH23 reversed both the calcofluor dark and the deoxycholate sensitive phenotypes associated with RmF346 and RmG425 (Table 12). The result indicates that the detergent sensitivity phenotype of the Class II suppressor mutations (*sfx-2* and *sfx-3*) is dependent on the *exoZ* locus and not *fix114*. When pTH56, containing

TABLE 12. REVERSAL OF THE DEOXYCHOLATE SENSITIVE PHENOTYPE OF CLASS II SECOND SITE REVERTANTS OF *fix-114* BY pTH23

| Strains | Calc | DE |
|---|---|---|
| Rm1021 | bright | ++ |
| RmF114 | dark | ++ |
| RmF346 | dark | +/- |
| RmG425 | dark | +/- |
| RmF346(pTH56) | dark | +/- |
| RmF346(pTH23) | bright | ++ |
| RmG425(pTH56) | dark | +/- |
| RmG425(pTH23) | bright | + |

Abbreviations: Calc, LB agar supplemented with 0.2% calcofluor; DE, LB agar supplemented with 1 mg/ml deoxycholate and 0.25 mM EDTA; ++, wild type growth; +, poor growth (small colonies); +/-, poor growth no single colonies; -, no growth; bright, single colonies appeared bright when viewed with uv light; dark, single colonies appeared dark when viewed with UV light.

sfx-1, was introduced into each of the Class II suppressor strains RmF346 and RmG425 the deoxycholate phenotype was not reversed (Table 12). This indicates that multiple copies of sfx-1 cannot mask the phenotype associated with the sfx-2 and sfx-3 and that the two classes of suppressors are distinct.

## Second site revertants do not restore complete symbiotic phenotype on sweet clover

Changes in outer cell surface are known to show host specific effects (Borthakur et al. 1986, Walker et al. 1989, Diebold et al. 1989). To determine if the fix114 suppressor mutations were alfalfa specific the second site revertant strains were inoculated onto medicago sativa cv. Iroquois and melilotus alba cv. Polara. Statistical analysis (Tukey's multirange test and Student's t-test) confirms that there are no significant differences ($p = 0.05$) in the acetylene reduction and dry weight values on alfalfa which is consistent with visual observations. The same analysis on the acetylene reduction and dry weight values obtained with sweet clover inoculated with the fix114 second site suppressors indicates that the Class I second site revertant RmF263 is significantly different ($p = 0.05$) from both Class II second site revertants (RmF346 and RmG425) and wild type.

Furthermore suppression by Class II mutations can be subdivided into two groups: one which is significantly different ( p = 0.05) from the Class I mutant, RmF263, and the Fix$^-$ parental phenotype, and the other which is not significantly different ( p = 0.05) from the Fix$^-$ parent (Table 13). These results correlate well with what is observed visually. In three separate experiments melilotus alba cv. Polara inoculated with RmF346 consistently appeared to have a partial Fix phenotype. The leaves of these plants appeared to be pale green to yellow, indicative of an ineffective symbiotic relationship. RmG425, however, appeared to be more effective when scored visually with no obvious visual differences. This correlates well with the values obtained by using the acetylene reduction assay and by the values obtained from the dry weight analysis on these same plants. This apparent difference in the effectiveness between RmF346 and RmG425 on sweet clover is also reflected in the number of nodules elicited. RmF346, which appears to be less symbiotically proficient than RmG425 appeared to elicit more nodules then the wild type (Table 14) which is typical of some other ineffective associations. As well it should be noted that RmG425 appears to elicited the same number of nodules per plant as wild type while Rm5408 and RmF114 elicited fewer nodules than the wild type Rm1021 when inoculated on sweet clover (Table 14).

TABLE 13. SYMBIOTIC PHENOTYPE OF SECOND SITE REVERTANTS ON ALFALFA AND SWEET CLOVER

A)

alfalfa

| Strains | ARA nM/plant/h ± SE | % wild type ARA | Dry Wt mg/plant ± SE | % wild type Dry Wt |
|---|---|---|---|---|
| Rm1021 | 378.8±23.3[a] | 100 | 12.3±2.2[a] | 100 |
| RmF114 | 120.9±19.3[b*] | 32 | 6.7±0.8[b] | 54 |
| Rm5408 | 85.6±21.5[b*] | 23 | 5.4±0.5[b,c] | 44 |
| RmF263 | 299.3±98.9[a] | 79 | 10.3±0.4[a] | 84 |
| RmF346 | 325.0±56.2[a] | 86 | 9.2±0.4[a] | 74 |
| RmG425 | 285.3±118.6[a] | 75 | 11.6±0.8[a] | 95 |
| U.I. | 3.3±0.5[c] | 1 | 4.7±0.3[c] | 39 |

Alfalfa (A) and sweet clover (B) were inoculated with second site revertants and harvested at 23 and 28 days respectively. Dry weights and acetylene reduction assays were performed as described in Methods.

Superscripts on ARA values on alfalfa (A) and sweet clover (B) correspond to statistical groupings using Tuckey's multirange test at the 95% confidence level. Superscripts on dry weight values on alfalfa (A) and sweet clover (B) correspond to statistical groupings using Students t-test at the 95% confidence level. Values with an equivalent superscript within columns on alfalfa (A) and sweet clover (B) show no significant differences.

* Second site revertants were isolated from these treatments.

TABLE 13. SYMBIOTIC PHENOTYPE OF SECOND SITE REVERTANTS ON ALFALFA AND SWEET CLOVER

B)

Sweet clover

| Strains | ARA nM/plant/h ± SE | % wild type ARA | Dry Wt mg/plant ± SE | % wild type Dry Wt |
|---|---|---|---|---|
| Rm1021 | 404.9±35.7[a] | 100 | 11.7±2.5[a] | 100 |
| RmF114 | 3.11±1.6[d] | <1 | 3.9±0.7[d,e] | 34 |
| Rm5408 | 3.0±0.9[d] | <1 | 4.7±0.2[d,e] | 40 |
| RmF263 | 249.7±25.4[b] | 62 | 6.2±0.2[b] | 53 |
| RmF346 | 87.0±16.2[c,d] | 21 | 5.2±0.4[d] | 48 |
| RmG425 | 125.8±35.5[c] | 31 | 5.7±0.2[c] | 45 |
| U.I. | 0.05±0.0[d] | <1 | 3.8±0.9[e] | 32 |

TABLE 14. NUMBER OF NODULES PER PLANT ELICITED BY SECOND SITE REVERTANTS ON SWEET CLOVER

| Strain | | Number of root systems examined | Number of nodules/root system ± SE |
|---|---|---|---|
| Rm1021 | wild type | 17 | 9.71 ± 0.99[a] |
| Rm5408 | Δ5408 | 25 | 5.04 ± 0.62[b] |
| RmF114 | ΔF114 | 17 | 4.00 ± 0.59[b] |
| RmF263 | *sfx-1*, Δ5408 | 15 | 9.47 ± 0.75[a] |
| RmF346 | *sfx-2*, ΔF114 | 14 | 14.60 ± 1.80[c] |
| RmG425 | *sfx-3*, ΔF114 | 19 | 10.42 ± 1.05[a] |
| Uninoculated | | 20 | 0 |

Sweet clover inoculated with second site revertants were harvested at 28 days, the root systems were separated and the number of nodules per root system was determined by counting nodules at 10X magnification. Results are presented as mean ± standard error. Values with an equivalent superscript show no significant differences using Students t-test at the 95% confidence level.

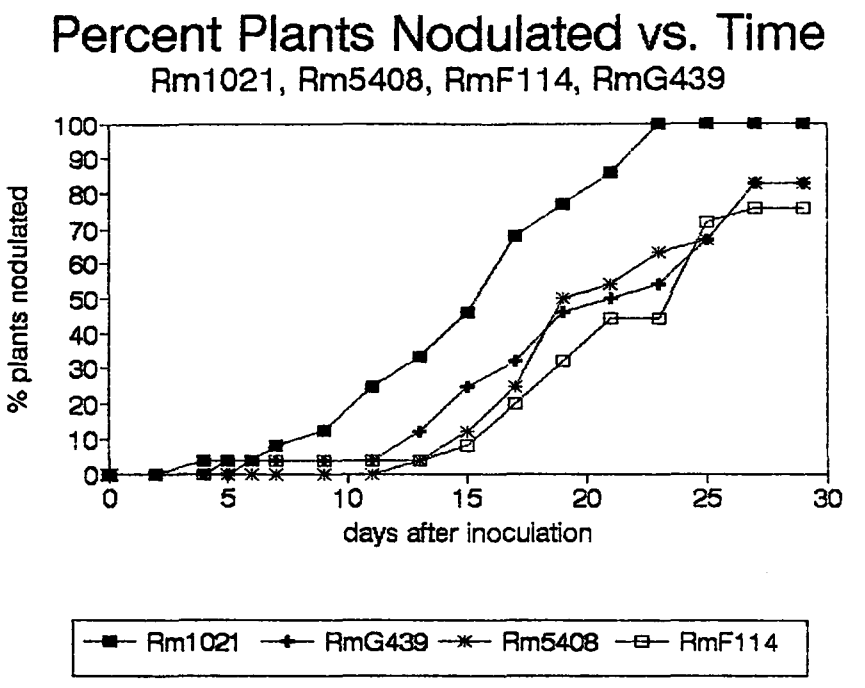
## Second site revertants do not restore wild type nodulation kinetics

Strains carrying ΔF114 or Δ5408 were previously observed to induce fewer nodules than wild type when inoculated onto alfalfa in Leonard pot assemblies (T. Charles personal communication). To determine whether the nodulation kinetics of strains carrying the second site revertants were altered relative to the parental deletion strains an experiment to quantify the nodulation kinetics was carried out (Figure 15, 16, 17).

The results show that Rm5408, RmF114, and RmG439 are all delayed in nodule formation by at least two days relative to Rm1021 and they produce fewer nodules than Rm1021 (Figure 15).

RmF346 and RmF263 both show an initial delay relative to Rm1021. Unlike RmF114 and Rm5408, the parental strains, the second site revertants attained 100% nodulation within 30 days. The fix114 suppressor mutations in RmF263 and RmF346, however, clearly did not restore the delay in nodulation to wild type levels (Figure 16, 17).

Figure 15. Nodulation kinetics of Rm1021 (wild type) and three strains, Rm5408, RmF114, and RmG439, each carrying deletions which remove *fix-114*.


Percent Plants Nodulated vs. Time
Rm1021, Rm5408, RmF114, RmG439
% plants nodulated
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
days after inoculation
Rm1021
RmG439
Rm5408
RmF114

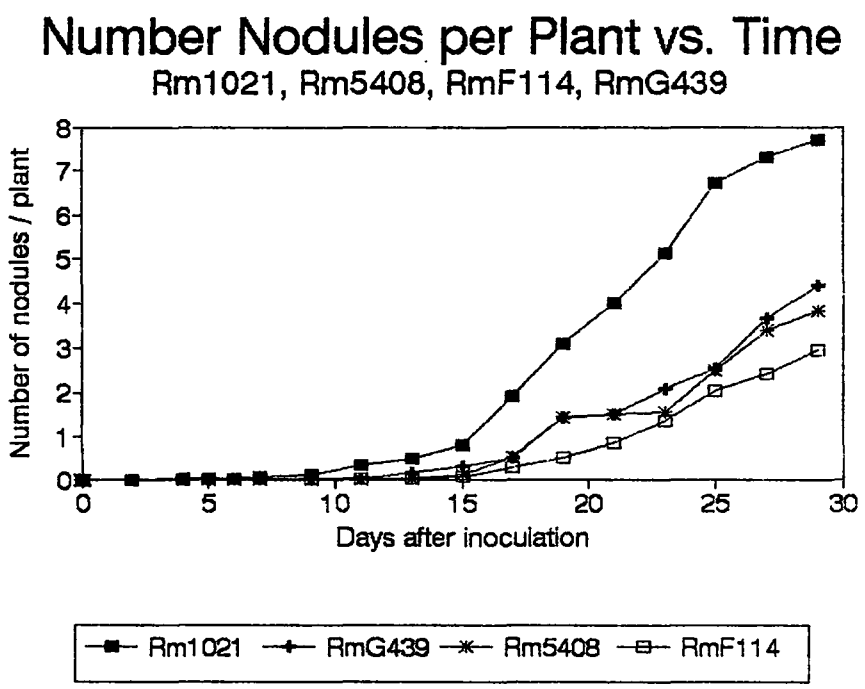
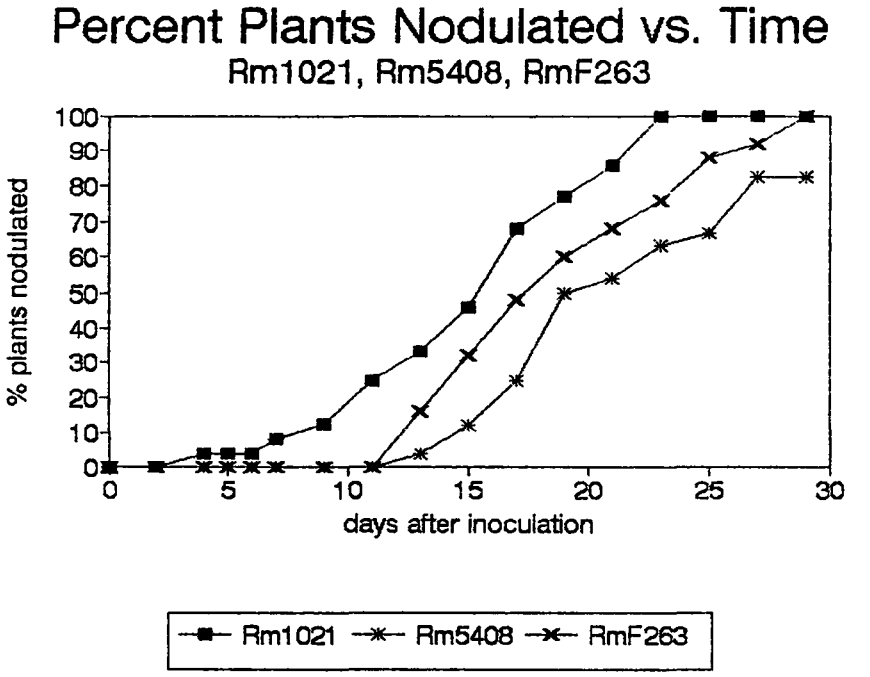

Number Nodules per Plant vs. Time
Rm1021, Rm5408, RmF114, RmG439
Number of nodules / plant
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
Days after inoculation
Rm1021
RmG439
Rm5408
RmF114

Figure 16. Nodulation kinetics of RmF263 carrying sfx-1 compared to the nodulation kinetics of the parental Fix⁻ strain Rm5408 and the wild type strain Rm1021.


Percent Plants Nodulated vs. Time
Rm1021, Rm5408, RmF263
% plants nodulated
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
days after inoculation
Rm1021
Rm5408
RmF263

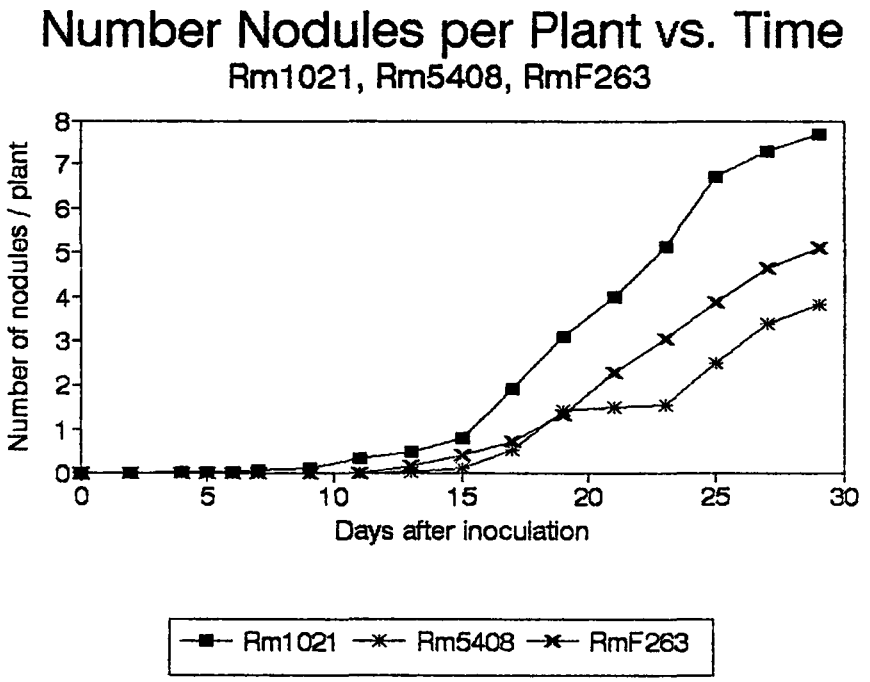
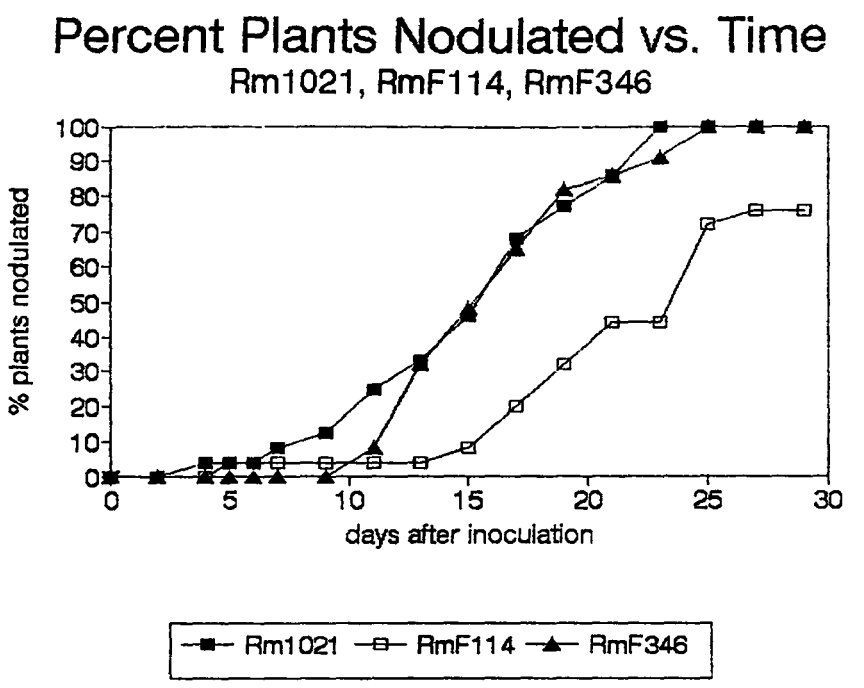
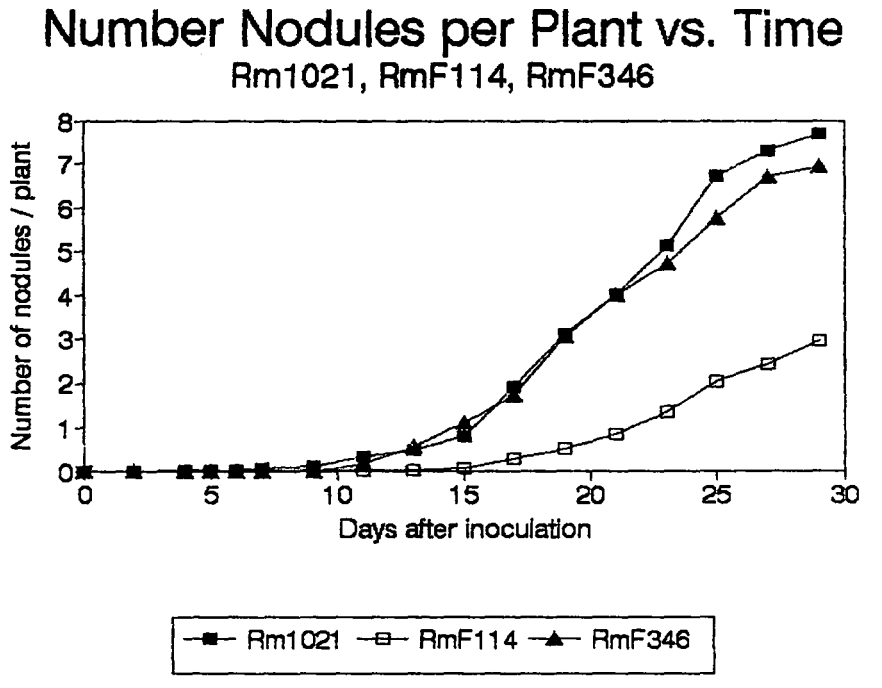

Number Nodules per Plant vs. Time
Rm1021, Rm5408, RmF263
Number of nodules / plant
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
Days after inoculation
Rm1021
Rm5408
RmF263

Figure 17. Nodulation kinetics of RmF346 carrying sfx-2 compared to the nodulation kinetics of the parental Fix$^-$ strain RmF114 and to the wild type strain Rm1021.


Percent Plants Nodulated vs. Time
Rm1021, RmF114, RmF346
% plants nodulated
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
days after inoculation
Rm1021
RmF114
RmF346


Number Nodules per Plant vs. Time
Rm1021, RmF114, RmF346
Number of nodules / plant
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
Days after inoculation
Rm1021
RmF114
RmF346

## sfx-1 and sfx-2 suppress the osmotic phenotype of fix114 when present as a single chromosomal copy

To determine whether the sfx-1 and the sfx-2 mutations suppressed the osmotic phenotype of fix114, constructs were made by transduction that contained single Fix$^-$ Tn5 inserts from the fix114 locus and either sfx-1 or sfx-2 as chromosomal copies in an otherwise wild type background. The results (Table 15) show that all single inserts in the fix114 locus as well as an 8 kb deletion of the fix114 locus are suppressed sfx-1 and sfx-2 on low osmolarity media and appear dry like the wild type strain Rm1021.

## sfx-1 and sfx-2 do not correspond to ndvB symbiotic pseudorevertants

R. meliloti ndvB mutants form Fix$^-$ nodules on alfalfa and appear to be unable to osmoadapt (Dylan et al. 1990). Dylan et al. (1990b) reported symbiotic pseudorevertants of ndvB mutants which were isolated from rare Fix$^+$ nodules. Because fix114 appeared to have an osmotic phenotype it was of interest to determine whether either sfx-1 or sfx-2 could suppress the ndvB Fix$^-$ symbiotic phenotype and thus be potentially allelic to the pseudorevertant mutations which were reported by Dylan et al. (1990b).

TABLE 15. REVERSAL OF THE LOW OSMOLARITY PHENOTYPE OF *fix114* MUTANTS BY *sfx-1* AND *sfx-2*

| Strain | Relevant Characteristics | 1/2 GYM |
|---|---|---|
| Rm1021 | Wild Type | dry |
| RmG439 | 12 kb deletion of *fix114* | mucoid[a] |
| RmF994 | Rm1021, *sfx-1*, Ω5118::Tn*5*-233 | dry |
| RmG479 | Rm1021, *sfx-2*, Ω5025::Tn*5* | dry |
| RmF919 | *fix114-1*::Tn*5* | mucoid |
| RmF921 | *fix114-4*::Tn*5* | mucoid |
| RmF922 | *fix114-5*::Tn*5* | mucoid |
| RmG156 | *fix114-1*::Tn*5*, *sfx-1* | dry |
| RmG158 | *fix114-4*::Tn*5*, *sfx-1* | dry |
| RmG159 | *fix114-5*::Tn*5*, *sfx-1* | dry |
| RmG514 | 12 kb deletion of *fix114*, *sfx-2* | dry |

Strains RmG156, RmG158, and RmG159 were constructed by transducing Tn*5* from RmF919, RmF922, and RmF926 into RmF994 carrying *sfx-1* and Ω5118::Tn*5*-233. RmG514 was constructed by transducing a 12 kb deletion of *fix-114* marked with $Sp^r$ from strain RmG424 into RmG479 containing Ω5025::Tn*5*.

[a] Mucoid phenotype of all *fix114* mutants was also reversed by the addidtion of 100 mM NaCl to the media.

A strain which carries an ndvB::Tn5 mutation in Rm1021 was constructed by transduction (Williams et al. 1989). A Tn5-233 derivative of this RmG588 (obtained from J. Reed, original designation Rm6027) was transduced into RmF379 and RmG479, which contain sfx-1 and sfx-2 respectively in a wildtype background, to construct double mutants. The position of the ndvB mutation has been mapped as being counter-clockwise from the trp-33 chromosomal marker on Meade and Signer's (1977) map of Rm1021 (J. Glazebrook personal communication). Since the position of the sfx-2 allele was not known precisely the ndvB::Tn5 marker from RmG588 was also transduced into RmG640. Strain RmG640 carries Ω5263::Tn5-233, which was isolated as being 70% linked to the sfx-2 region (Table 10). Fifty transductants were screened and no linkage was detected suggesting that ndvB::Tn5-233 was not linked to this region. Two transductants, which contained either sfx-1 and ndvB or sfx-2 and ndvB, from each construction were isolated and were designated RmG635, RmG636, RmG637, and RmG638. These strains were then tested for colony morphology, motility, and symbiotic proficiency on alfalfa (Table 17, 18, 19).

RmG637 and RmG638 containing sfx-2 and the ndvB mutation formed a dry/small colony on LB agar. When these constructs were streaked onto 1/2 GYM, TY, or YEM agar they

appeared to be sensitive and no single colonies were observed (Table 17). Moreover, when these constructs were tested for motility the results indicated that there is a cumulative effect of combining either sfx-1 or sfx-2 with the ndvB mutation in Rm1021 (Table 18). It is interesting to note that the ndvB phenotype in R. meliloti 102F34 was reported to have a phenotype that could be distinguished from wild type on 1/2 GYM agar and being on the basis of motility. The ndvB mutation in a Rm1021 background is indistinguishable from wild type on 1/2 GYM agar (Table 17) and shows only a slight reduction in motility (Table 18). However, when the sfx-1 or sfx-2 allele is added to the ndvB mutation in a Rm1021 background we see an extreme non-motile phenotype (Table 18), and when the sfx-2 allele is added we see a no growth phenotype on 1/2 GYM agar. One possibility may be that R. meliloti 102F34 carries a sfx-2 type allele. Inoculation of these constructs onto alfalfa resulted in a severe Fix$^-$ phenotype (Table 19). These data strongly suggests that sfx-1 and sfx-2 are not the same as the ndvB Fix$^-$suppressors previously described by Dylan et al. (1990b).

TABLE 16. REVERSAL OF *fix114* MOTILITY PHENOTYPE BY *fix114* SUPPRESSOR MUTATIONS

| Strains | | 27 hours Diameter of Swarm (mm ± SE) | 45 hours Diameter of Swarm (mm ± SE) |
|---|---|---|---|
| Rm1021 | wild type | 25.0 ± 0.58 | 37.33 ± 0.33 |
| Rm5408 | Δ5408 | 15.0 ± 0.0 | 25.0 ± 1.15 |
| RmF114 | ΔF114 | 14.33 ± 2.33 | 26.67 ± 0.33 |
| RmF263 | *sfx-1*, Δ5408 | 24.33 ± 1.20 | 34.0 ± 1.53 |
| RmG203 | *sfx-4*, Δ5408 | 24.67 ± 0.33 | 35.67 ± 0.88 |
| RmG204 | *sfx-5*, ΔF114 | 24.0 ± 1.53 | 35.33 ± 2.33 |
| RmF346 | *sfx-2*, ΔF114 | 22.33 ± 0.88 | 32.67 ± 1.67 |
| RmG425 | *sfx-3*, ΔF114 | 19.67 ± 1.85 | 31.0 ± 3.22 |

10 μl of overnight cultures were spotted onto Yeast Extract Swarm media and measurements were taken at 27 and 45 hours. Data is presented as the mean of three replicates ± Standard Error.

TABLE 17. EFFECTS OF THE fix114 SUPPRESSOR MUTATIONS ON THE ndvB PHENOTYPE

| Strains | Relevant Characteristics | 1/2 GYM | 1/2 GYM 100mM NaCl | LB | YEM | TY |
|---|---|---|---|---|---|---|
| Rm1021 | Wild Type | ++ | ++ | ++ | ++ | ++ |
| RmF379 | sfx-1 | ++ | ++ | ++ | ++ | ++ |
| RmG479 | sfx-2 | ++ | ++ | ++ | ++ | ++ |
| RmG588 | ndvB | ++ | ++ | ++ | ++ | ++ |
| RmG635 | sfx-1, ndvB | ++ | ++ | ++ | ++ | ++ |
| RmG636 | sfx-1, ndvB | ++ | ++ | ++ | ++ | ++ |
| RmG637 | sfx-2, ndvB | - | ++ | ++[a] | +/-[b] | +/- |
| RmG638 | sfx-2, ndvB | - | ++ | ++[a] | +/-[b] | +/- |

++, good growth, +, poor growth small colonies, +/-, no isolated single colonies, -, no growth. Media used are described in Materials and Methods section.

[a] good growth but colonies had a dry/small phenotype

[b] primary streak was clearly mucoid, no isolated single colonies.

TABLE 18. EFFECTS OF *fix114* SUPPRESSOR MUTATIONS ON *ndvB* MOTILITY PHENOTYPE

| Strain | 24 hours Diameter of Swarm (mm ± SE) | 44 hours Diameter of Swarm (mm ± SE) |
|---|---|---|
| Rm1021 (wild type) | 19.0 ± 0.0 | 32.33 ± 0.58 |
| RmF379 (*sfx-1*) | 21.0 ± 0.0 | 33.33 ± 0.58 |
| RmG479 (*sfx-2*) | 21.0 ± 1.0 | 33.0 ± 1.0 |
| RmG588 (*ndvB*) | 17.67 ± 0.58 | 25.33 ± 0.58 |
| RmG635 (*sfx-1*, *ndvB*) | 14.33 ± 0.58 | 19.0 ± 1.0 |
| RmG637 (*sfx-2*, *ndvB*) | 12.0 ± 0.0 | 17.33 ± 0.58 |

10 $\mu$l of an overnight culture was spotted onto Yeast Extract swarm media and measurements were made at approximately 24 hour intervals. Numbers are expressed as the mean of three replicates ± Standard Error.

TABLE 19. SYMBIOTIC EFFECT OF SECOND SITE REVERTANTS TO *fix114* ON A *ndvB* MUTANT OF RM1021

| Strain | Relevant Characteristics | ARA[a] nM/plant/h ± SE | % wild type ARA | Dry Wt mg/plant ± SE | % wild type dry wt |
|---|---|---|---|---|---|
| Rm1021 | wild type | 544.7 ± 49.8 | 100 | 13.1 ± 2.9 | 100 |
| RmF379 | *sfx-1* | 551.6 ± 157.6 | 101 | 13.1 ± 0.9 | 100 |
| RmG479 | *sfx-2* | 447.5± 57.6 | 82 | 13.9 ± 1.7 | 100 |
| RmG588 | *ndvB* | 7.6 ± 2.8 | 1 | 5.3 ± 0.1 | 40 |
| RmG635 | *sfx-1*, *ndvB* | 8.9 ± 0.4 | 2 | 4.5 ± 0.5 | 34 |
| RmG637 | *sfx-2*, *ndvB* | 10.7 ± 0.0 | 2 | 5.9 ± 0.5 | 45 |
| U. I.[b] | | 10.7 ± 0.0 | 2 | 5.9 ± 0.4 | 45 |

Alfalfa plants were 24 days old when they were assayed for acetylene reduction and dry weight. Values presented are the mean ± standard error of three replicates consisting of 3 plants per replicate for acetylene reduction values and between 7-9 plants per replicate for dry weight analysis.

[a] Acetylene reduction activity

[b] Uninoculated control

sfx-1 and sfx-2 do not suppress EPS-I mutations

Because RmF346 and RmG425 carrying sfx-2 and sfx-3 respectively showed phenotypes that appeared to be indicative of alterations in the outer cell membrane, we wished to examine whether any of the second site mutations which suppress the Fix phenotype associated with the fix114 locus could also suppress the Fix$^-$ phenotype associated with EPS mutations in Rm1021.

EPS mutations, exoA31::Tn5, exoB13::Tn5, and exoF55::Tn5 from strains Rm7031, Rm7013, and Rm7055 respectively were introduced into RmF263, RmF346, and RmG425 by transduction. The resultant double mutants were single colony purified three times and then tested for sensitivity to deoxycholate, fluorescence on calcofluor media and symbiotic proficiency on alfalfa (Table 20). The results show that combining the second site fix114 suppressors with either exoA31 (strains RmG657-659), exoB13 (strains RmG660-662), or exoF55 (RmG663-665) had no apparent effect on the calcofluor dark phenotype or the Fix phenotype, but the combination of exoB13 or exoF55 with either sfx-2 or sfx-3 in RmF346 and RmG425 respectively reversed the deoxycholate phenotype associated with RmF346 and RmG425 (Table 20). This was not the case when exoA31 was introduced into RmF346 or RmG425. In contrast, the combining of either exoA31, exoB13, or exoF55 with sfx-1 in RmF263 did not have any

effect on any of the phenotypes which were examined (Table 20).

These results seem to indicate that although the second site revertants of fix114 do not suppress the Fix$^-$ phenotype associated with the EPS mutants, some type of complex interaction between exoB13 and exoF55 is occuring that allows these strains to be resistant to deoxycholate. It is not clear at this time what these interactions are.

TABLE 20. EFFECT OF EPS MUTATIONS ON SECOND SITE *fix114* SUPPRESSORS

| STRAIN | RELEVANT CHARACTERISTICS | CALC | DE[a] | FIX[b] |
|---|---|---|---|---|
| Rm1021 | Wild type | bright | ++ | + |
| Rm7031 | *exoA31*::Tn*5* | dark | ++ | - |
| Rm7013 | *exoB13*::Tn*5* | dark | ++ | - |
| Rm7055 | *exoF55*::Tn*5* | dark | ++ | - |
| RmF114 | ΔΩ5033-5064::Tn*5*-233 (ΔF114) | dark | ++ | - |
| RmF263 | Δ5408, *sfx-1* | dark | ++ | + |
| RmG657 | Δ5408, *sfx-1*, *exoA31*::Tn*5* | dark | ++ | - |
| RmG660 | Δ5408, *sfx-1*, *exoB13*::Tn*5* | dark | ++ | - |
| RmG663 | Δ5408, *sfx-1*, *exoF55*::Tn*5* | dark | ++ | - |
| RmF346 | ΔF114, *sfx-2* | dark | - | + |
| RmG658 | ΔF114, *sfx-2*, *exoA31*::Tn*5* | dark | +/- | - |
| RmG661 | ΔF114, *sfx-2*, *exoB13*::Tn*5* | dark | ++ | - |
| RmG663 | ΔF114, *sfx-2*, *exoF55*::Tn*5* | dark | ++ | - |
| RmG425 | ΔF114, *sfx-3* | dark | +/- | + |
| RmG659 | ΔF114, *sfx-3*, *exoA31*::Tn*5* | dark | + | - |
| RmG662 | ΔF114, *sfx-3*, *exoB13*::Tn*5* | dark | ++ | - |
| RmG665 | ΔF114, *sfx-3*, *exoF55*::Tn*5* | dark | ++ | - |

Strains RmG657-RmG665 were constructed by transducing Tn*5* from strains Rm7031, Rm7013, and Rm7055 into each of RmF263, RmF346, and RmG425. Transductants were single colony purified and tested on LB agar supplemented with 0.2% calcofluor (Calc), LB agar supplemented with 1 mg/ml deoxycholate and 0.25 mM EDTA (DE). Strains were also inoculated onto alfalfa to test for symbiotic phenotype. Abbreviations: bright, single colonies appeared bright when viewed with uv light; dark, single colonies appeared dark when viewed with uv light.

[a] +++, good growth like wild type, +, poor growth small colonies, +/-, no isolated single colonies, -, no growth.

[b] +, dry weights of plants were similar to wild-type and plants appeared $Fix^+$, -, dry weights of plants were similar to uninoculated controls and plants appeared $Fix^-$.

# CHAPTER 6

## DISCUSSION

### The Fix⁻ Phenotype of ΔF114 and Δ5408 can be Suppressed by Second Site Mutations

Rm5408 and RmF114 are Fix⁻ because they carry Δ5408 and ΔF114 respectively both of which delete the *fix114* region. *Rhizobia* isolated from Fix⁺ alfalfa nodules following inoculation with RmF114 or Rm5408 carry second site mutations which suppress the Fix⁻ phenotype of Rm5408 and RmF114. There are three lines of evidence which support this conclusion: 1) Bacteria isolated from Fix⁺ nodules occurring on alfalfa inoculated with RmF114 or Rm5408 were Fix⁺ when reinoculated onto alfalfa. This showed that the newly acquired Fix⁺ phenotype was stable (Table 2, Table 13). 2) All isolates retained the Tn*5*-233 insertion which marked the ΔF114 and Δ5408. All isolates were also dark on calcofluor plates and could not utilize dulcitol as a sole carbon source. In the case of RmF263 it was demonstrated that when the Tn*5*-233 marker was transduced into Rm1021 that the Fix⁻ phenotype was co-transduced (20/20 scored). All of these phenotypes are associated with ΔF114 and Δ5408. 3) The mutations causing the suppression of Fix⁻ in Rm5408 and

RmF114 readily arose, could be manipulated genetically, and mapped to one of two loci. Together these suggest that the suppression of the Fix⁻ phenotype is due to a single second site mutation.

## Mapping and Cloning of sfx-1

The majority of the work reported in this thesis was carried out using RmF263 which carries Δ5408 and the sfx-1 allele. This allele was manipulated such that Tn5 inserts linked to the second site mutation (sfx-1) were isolated (Figure 4). Two Tn5 inserts, Ω5117::Tn5 and Ω5118::Tn5 in RmF338 and RmF339 respectively were isolated in this manner. Tn5-132 markers that were subsequently isolated as being linked in transduction to Ω5118 were also found to be linked to Ω5117 (Figure 5) suggesting that both markers were in the same genetic region. A three factor cross showed that the sfx-1 allele was linked to Ω5118 (Figure 4). Subsequent conjugal mapping of Tn5-233 replacements of Tn5 insertions Ω5117 and Ω5118 demonstrated that these markers are linked to the chromosomal marker trp-33 (Table 3). This showed that sfx-1, which suppresses a symbiotic mutation found on pRmeSU47b, is located on the chromosome of Rm1021.

To clone the sfx-1 allele we assumed that the sfx-1 allele might be dominant to the wild type allele. If this

were the case we could isolate a cosmid containing the sfx-1 allele by constructing a cosmid clone bank on the strain containing the sfx-1 (RmF263), mating the bank en masse into the Fix$^-$ parental strain Rm5408, and isolating bacteria from complemented Fix$^+$ nodules.

Two independent cosmid banks were constructed with DNA from RmF263 in the broad host range vector pRK7813 (Jones and Gutterson 1987). Isolation of bacteria from Fix$^+$ nodules from alfalfa that was inoculated with Rm5408 or RmF114 transconjugants containing the cosmid bank yielded two different cosmids: pTH56 and pTH57. Restriction analysis of these cosmids suggested that they had a single 18 kb BamH1 fragment in common. This was confirmed by isolating the 18 kb BamH1 fragment of pTH56 from a low melting point gel, labelling the isolated DNA and probing restricted pTH57 (Figure 11). As well, when a HindIII digest of pTH57 was probed with the 18 kb BamHI fragment from pTH56 we see that the 7 kb HindIII fragment which is found in pTH56 is missing and appears to be replaced by a 5 kb HindIII fragment in pTH57 (Figure 11). However on a double BamHI HindIII digest both pTH56 and pTH57 appear to have identical fragments hybridizing to the 18 kb BamHI fragment from pTH56 (Figure 11). Therefore it appears that pTH56 and pTH57 carry a single 18 kb BamHI fragment in common and that this fragment extends into a 7 kb HindIII

fragment which is intact in pTH56 but is truncated in pTH57. This suggests that the only common insert DNA between pTH56 and pTH57 is the 18 kb BamHI fragment and that these two cosmids extend in two different directions.

To demonstrate that pTH56 carried DNA that was contiguous with the sfx-1 region, total DNA from strains containing Tn5-233 insertions which were isolated and mapped between the insertions Ω5117 and Ω5122 were probed with labelled pTH56. Southern blot analysis showed that total DNA from RmG352, which contains Ω5234::Tn5-233, restricted with BamHI did not hybridize with the 7kb BamHI fragment of pTH56 but to a 13 kb BamHI fragment instead. Since BamHI does not cut within Tn5-233 (DeVos et al. 1987) this corresponds to the expected size if Ω5234::Tn5-233 were within the corresponding 7 kb BamHI fragment in RmG342. Similarly RmG351 containing Ω5244::Tn5-233 showed a shift in the 18 kb BamHI fragment (Figure 12). In addition an active TnphoA insertion, Ω21A, which was isolated from pTH56 were also mapped by a three factor transductional cross to the sfx-1 region following homogenotization (Figure 13). These results unambiguously show that the insert DNA in pTH56 is co-co-linear with the region which was genetically defined as containing the sfx-1 allele.

When pTH56 or pTH57 were reintroduced into either Rm5408 or RmF114 and inoculated onto alfalfa they reversed

the Fix$^-$ symbiotic phenotype associated with ΔF114 and Δ5408 (Table 4). Acetylene reduction activity and dry weight analysis, however, seem to vary from 40-121% and 66-94% respectively. This may be due to plasmid instability, depending upon what stage of nodulation the sfx-1 allele is required, or alternatively this could also be due to a co-dominant effect of sfx-1 to sfx-1$^+$. When pTH56 was introduced into RmG439 and other single Tn5 inserts in fix114 it also reversed the mucoid phenotype associated with fix114 mutations on low osmolarity media (Table 5). These results are consistent with pTH56 carrying the sfx-1 allele.

## Two Distinct Classes of fix114 Suppressor Mutations

The five independent second site revertants that were studied can be divided into two distinct classes: Class I consisting of RmF263 carrying sfx-1, RmG203 carrying sfx-4, and RmG204 containing sfx-5, and Class II consisting of RmF346 carrying sfx-2 and RmG425 carrying sfx-3. This division is based on mapping data and phenotypic differences between strains carrying these different alleles.

When the wild type sfx-1 region flanked by Ω5117 and Ω5122 was transduced into RmF263, RmF346, RmG203, and RmG204 it was found that RmF263, RmG203, and RmG204 lost their ability to suppress the Fix$^-$ phenotype whereas RmF346 still retained the ability to suppress the Fix$^-$ phenotype

associated with ΔF114. This indicated that the sfx-2 mutant allele found in RmF346 is not within the region defined by Ω5117 and Ω5122 (Figure 14). The possibility that a quadruple crossover event occurred was unlikely since all eight transductants (two transductants from each of four independent crosses) that were tested each gave the same result. This suggests that sfx-2 allele in RmF346 does not map to the same locus as sfx-1.

It was also found that RmF346 and RmG425 were sensitive to a variety of detergents and to the antibiotic bacitracin whereas strains carrying Class I mutations (RmF263, RmG203, and RmG204) as well as the parental Fix$^-$ strains, Rm5408 and RmF114, and the wild type were not sensitive to these same compounds (Table 9). This suggests that RmG425 may carry a second site suppressor which is allelic to sfx-2. It was later found that both sfx-2 in RmF346, and sfx-3 in RmG425 were each about 70% linked to Ω5258::Tn5 which was isolated as linked to the sfx-2 locus (Table 13). Together these data suggest that RmF346 and RmG425 carrying sfx-2, and sfx-3 respectively constitute a distinct locus separate from the locus represented by sfx-1. As well, the nodulation kinetics of RmF346 are different from those of RmF263 (Figure 16), and strains that contained sfx-2 and ndvB gave a different motility and plate phenotype than those which contained sfx-1 and ndvB (Table 17). These data reinforce

the idea that there are at least two distinct classes of second site revertants, Class I, those found between Ω5117 and Ω5122, and Class II second site revertants, those that appear sensitive to detergents and do not map to the region defined by Ω5117 and Ω5122.

Tn5-233 insertions linked to Ω5258::Tn5 in strain RmG558 were subsequently isolated. One of these inserts, Ω5263::Tn5-233 which was 60% linked in transduction to Ω5258::Tn5 was mapped by conjugation to the chromosome of Rm1021 (Table 11). The results showed that Ω5263::Tn5-233, like Ω5117::Tn5-233 and Ω5118::Tn5-233 which are linked to sfx-1, is also linked to trp-33 in a clockwise orientation. The transductional linkage between these markers, if any, is not known.

## Characterization of Second Site Revertants

The most predominant difference found between Class I and Class II second site revertants is the difference in sensitivity to detergents and to the antibiotic bacitracin (Table 9). The sensitivity to detergents however is dependent on the presence of the deletions Δ5408 or ΔF114 (T. Charles personal communication). pTH23, a cosmid that complements the Exo$^-$ (calcofluor dark) phenotype of ΔF114 (exoZ), was introduced into both Class II second site revertants RmF346 and RmG425 (Table 12). In addition to

reversing the calcofluor phenotype, pTH23 also reverses the deoxycholate phenotype associated with Class II revertants. This suggests that these two separate loci, *sfx-2* and *exoZ*, in RmF114 help determine the sensitivity to detergents. It appears that either the *sfx-2*$^{+}$ or the *exoZ* locus on pTH23 are needed for resistance to deoxycholate in a ΔF114 background. An interesting possibility is that these two loci may interact in some as yet unknown manner and at least one of these regions is needed for resistance to deoxycholate at the levels which were used.

Sensitivity to detergents is often associated with alterations in the outer cell membrane of *E. coli* and *S. typhimurium*. For example "deep rough" mutants of *E. coli* and *S. typhimurium* which produce LPS that is missing all of the O antigen and most of the core antigen (see Figure 1) are extremely sensitive to detergents (Sanderson *et al.* 1974). As well, it has also been reported that an *E. coli* mutant which is missing OmpA and another major surface protein from the outer cell membrane became sensitive to SDS, deoxycholate, and Triton X-100 (Scheizer *et al.* 1976). More recently a class of mutants of *S. typhimurium* were isolated that have a more permeable outer membrane but do not have an altered LPS, phospholipid, or any identifiable changes in the outer membrane protein profile. These mutants also show sensitivity to detergents (Sukupolvi *et*

al. 1984). It appears that many different mutations that are associated with the changes to the outer cell membrane can result in a detergent sensitive phenotype.

Bacitracin has been shown to specifically inhibit the dephosphorylation of a glycosyl carrier lipid *in vitro* (Siewert *et al.* 1967, Stone *et al.* 1968). The glycosyl carrier lipid appears to be involved both in the O antigen synthesis for LPS (Wright *et al.* 1967) as well as being involved in peptidoglycan synthesis (Higashi *et al.* 1967). Because Class II second site revertants are sensitive to both detergents and bacitracin it may be that the mutation alters the cell membrane in these mutants. This alteration may make the membrane more permeable to compounds that are normally excluded from the cell such as detergents and bacitracin.

Class I mutations, however, do not show sensitivity to these same compounds (Table 9). It is interesting to note that active TnphoA inserts that were isolated from pTH56 and mapped within 20 kb of *sfx-1* gave the same general phenotype on plates as Class II revertants indicative of alterations in the cell surface (Table 8). It is possible that although the *sfx-1* mutation does not have an obvious defect indicative of a cell surface alteration that this region may still be involved in synthesis of components associated with the outer cell membrane and thus be part of a larger gene

cluster. Clustering of genes in Rhizobium known to code for exopolycaccharide and LPS biosynthesis over extended regions of DNA has been reported. For example the genes encoding EPS I and EPS II in R. meliloti extend over 30 kb and 20 kb of DNA respectively (Long et al. 1988a, Glazebrook et al. 1989). Genes needed for complete LPS synthesis in R. leguminosarum biovars phaseoli and viciae also span at least 20 kb (Prieffer 1989, Cava et al. 1990).

Alterations in the cell surface are associated with changes in phage sensitivity in E. coli and Rhizobia (Hancock et al. 1976, Finan et al. 1985, Clover et al. 1989). Class I and Class II revertants, however, do not show any alterations in sensitivity to 9 different R. meliloti bacteriophage (Table 9).

## Class I and Class II mutants do not restore a complete Fix$^+$ symbiosis on sweet clover

When Class I and Class II mutants are inoculated onto sweet clover cv. Polara, the plants are less effective relative to plants inoculated with the wild type, Rm1021, with dry weight and acetylene reduction values consistently 50% or lower than that of wild type (Table 13). Statistical analysis of the acetylene reduction data using Tukey's multirange test and of the dry weight values using Student's t-test showed that there are no significant differences

between Class I and Class II revertants when inoculated on alfalfa. However, when the same analysis was carried out on the acetylene reduction values and the dry weight values of sweet clover which were inoculated with Class I and Class II mutants significant differences were found (Table 11). Sweet clover inoculated with the Class I revertant, RmF263, showed nitrogenase levels of approximately 60%, as determined by the acetylene reduction assay and plants had approximately 50% of the dry weight of plants inoculated with Rm1021. Sweet clover inoculated with Class II mutants appeared to give acetylene reduction values which were about half of Class I second site revertants and dry weight values that were consistently lower than those of the Class I mutant RmF263. Statistical analysis of the acetylene reduction values using Tukey's multirange analysis and of the dry weight values using Student's t-test further subdivided Class II mutants into two groups. The activity of one of these, RmF346 carrying sfx-2, was not significantly different from the parental Fix$^-$ strains RmF114 and Rm5408. This co-relates well with what is observed visually.

Quantification of the nodules elicited by the second site revertants on sweet clover revealed that plants that were inoculated with RmF346 elicited significantly more nodules than plants that had been inoculated with wild type

(Table 14). Sweet clover inoculated with RmF346 has an average of 5 more nodules per plant than those that have been inoculated with Rm1021, RmF263, or RmG425 (Table 14). Ineffective strains often elicit more nodules than effective strains (Nutman 1952). It should be noted, however, that the nodules elicited by RmF346 on sweet clover did appear to be light pink and appeared to have some nitrogenase activity as measured by the acetylene reduction assay (Table 13).

It is generally believed that levels of fixed nitrogen can influence the nodulation of legumes by rhizobia. Thus it may be that the levels of fixed nitrogen in nodules formed by RmF346 is below a threshold level and the plant may not inhibit further nodule development. RmF346 appears to elicit close to wild type levels of nodules on alfalfa. In addition, these nodules appeared to fix nitrogen comparable to alfalfa inoculated with wild type (Figure 17, Table 13).

It has been reported that some of the LPS antigens in Rhizobium change within the bacteroid (Vanden Bosch et al. 1989). There is also evidence that bacteroids are more sensitive to detergents such as Triton X-100 and deoxycholate than free living bacteria (Sutton and Patterson 1980). This may suggest that bacteroids have a very different outer membrane than free living bacteria.

The phenotype of Class II second site revertants is indicative of changes occurring in the outer cell surface that allow these mutants to become symbiotically effective. Such a change can be envisioned as either a loss or a gain of function(s) that allows the bacteria to overcome the original symbiotic block. However, such a change does not necessarily mean that this change will be beneficial for symbiosis on other hosts. Sutton and Patterson (1980) suggested that the host plant is responsible for the degree of sensitivity to detergents of the bacteroid. If such were the case, this may suggest that the plant may be determining what changes occur in the outer membrane of the bacteria during symbiosis. Therefore a change that is beneficial on one host need not necessarily be beneficial on another host. This may help explain why we see differential suppression when second site revertant strains are inoculated on sweet clover.

## Nodulation Kinetics of Second Site Revertants

The second site revertants in this study (RmF263, RmF346, RmG203, RmG204, and RmG425) were isolated from $Fix^+$ nodules on their ability to suppress the $Fix^-$ phenotype associated with *fix114* in strains Rm5408 and RmF114. Another phenotype that was associated with Rm5408 and RmF114 was a reduced motility on Yeast Extract swarm media. This

phenotype was suppressed by all the second site revertants (Table 16). Because the second site revertants did not restore symbiosis completely on sweet clover it was of interest to further investigate the symbiotic phenotype on alfalfa. To determine if the symbiotic defect associated with ΔF114 and Δ5408 was completely reversed, a nodulation kinetics experiment was carried out.

The results show that both RmF114 and Rm5408 which carry large defined deletions of approximately 150 kb removing fix114 show a delay in the appearance of nodules by at least 2-3 days, and that RmG439 which carries a smaller defined deletion of 12 kb which removes fix114, also shows this delay. The interpretation of this is that removal of fix114 results in a delay of nodulation. When the Class I revertant RmF263 (carrying sfx-1) (Figure 16) and the Class II revertant RmF346 (carrying sfx-2) (Figure 17) were examined it was found that neither of the second site revertants reverse the delay which is characteristic of a fix114 deletion. Although the delay of nodulation is not reversed by the second site revertants it appears that both RmF263 and RmF346 are better nodulators than their parental strains Rm5408 and RmF114. Both RmF263 and RmF346 attained 100% nodulation in these experiments which contrasts with the parental strains which only achieved approximately 70% (Figure 16, Figure 17). As well, it would appear that both

RmF263 and RmF346 elicited more nodules per plant than the parental Fix$^-$ strains Rm5408 and RmF114. RmF263 appeared to give a value of 5 nodules per plant which appears intermediate between the parental Fix$^-$ strain Rm5408 and the wild type strain Rm1021 where as RmF346 appears to elicit as many nodules per plant as the wild type.

Although the second site revertants did not reverse the delay which is characteristic of their parental strains they clearly have an effect on the efficiency of nodulation (ie. number of nodules/plant). The results suggest that the suppression by the second site mutations sfx-1, and sfx-2, in RmF263 and RmF346, respectively, appear to be different and that the mechanism of suppression is not a direct functional replacement of the deleted fix114 locus because the delay in nodulation is not altered.

## Second Site Revertants do Not Suppress ndvB

R. meliloti ndvB mutants do not produce cyclic $\beta$ (1,2) glucan and have phenotypes that appear very similar to phenotypes associated with fix114 mutants (Dylan et al. 1986, Dylan et al. 1989b). For example, single Tn5 inserts and deletions of fix114 have a low osmolarity dependent phenotype. Strains containing deletions ΔF114 and Δ5408 have a motility phenotype. Both appear to form empty nodules on alfalfa. As well, second site revertants which

reverse the symbiotic phenotype have been isolated. All of these phenotypes have been reported for ndvB mutants in R. meliloti (Dylan et al. 1986, Dylan et al. 1990a, Dylan et al. 1990b).

Second site revertants of strains containing Δ5408 and ΔF114 reverse both the motility and symbiotic phenotypes associated with these deletions (Table 2, Table 16). pTH56 which contains sfx-1 suppresses the mucoid phenotype of all fix114 insertions tested (Table 5). When strains were constructed that contained sfx-1 or sfx-2 in an otherwise wild type background double mutants were constructed which contained fix114 inserts and either sfx-1 or sfx-2. It was found that when sfx-1 or sfx-2 were present as chromosomal copies they could suppress the mucoid phenotype associated with fix114 insertions on low osmolarity media (Table 15).

Because of the similarities in the phenotypes between fix114 and ndvB, the ndvB mutation was introduced into strains which contained sfx-1 or sfx-2 in a wild type background. It was found that second site revertants of ΔF114 and Δ5408 did not suppress the Fix$^-$ phenotype of ndvB on alfalfa (Table 19). In fact when ndvB is introduced into a strain containing the sfx-2 allele the resultant strains appear to be severely compromised for growth on different media and in motility (Table 17, Table 18). The strains carrying ndvB and sfx-2 did not grow on 1/2 GYM, TY, and YEM

agar but did grow on LB or 1/2 GYM supplemented with 100 mM NaCl. Although there are many differences in the media tested, media on which these strains appeared sensitive had very little added NaCl. LB contains 86 mM NaCl whereas 1/2 GYM, TY, and YEM agar have very little if any added NaCl. In light of this, the extreme motility phenotype (Table 18) may also be due to an osmolarity or salt effect since yeast extract swarm media is also low in added NaCl. Strains containing ndvB and sfx-1 appear to behave quite differently on the same media (Table 17), but they are also reduced in motility (Table 18). It is not readily apparent why this may occur. It is interesting to note, however, that each of the mutations on their own are not severely effected on the same media and this therefore seems to be a cumulative effect.

Second Site Revertants do Not Suppress exo Mutations

It has recently been reported that Fix$^-$, exo mutations (exopolysaccharide deficient) have been suppressed by alterations in other polysaccharide determinants found on the outer envelope of R. meliloti. In R. meliloti, Rm1021, mutations which affect biosynthesis of the succinoglycan binding exopolysaccharide, EPS I, can be suppressed by the expression of a second cryptic exopolysaccharide that is found on megaplasmid pRmeSU47b of Rm1021 (Glazebrook et al.

1989, Zahn et al. 1989). It appears that all mutants except exoB mutations could be suppressed by EPS II (Glazebrook et al. 1989). It has also been reported that AK631, a derivative of R. meliloti Rm41, carries an exoB type mutation but is symbiotically proficient due to a second locus lpsZ which is located on a megaplasmid which is able to mask the exopolysaccharide deficiency (Williams et al. 1990). This locus, lpsZ does not appear to occur in Rm1021 but transfer of the megaplasmid from Rm41 into Rm1021 results in the suppression of the exoB symbiotic phenotype (Williams et al. 1989). In Rm41, it appears that in addition to having lpsZ, LPS generally has the same function as exopolysaccharide (Putnoky et al. 1990).

Because of the apparent interaction of sfx-2 and sfx-3 in RmF346 and RmG425 respectively with the exoZ locus (Table 12) it was of interest to determine whether the second site revertants to fix114 could suppress the symbiotic deficiency associated with various EPS mutants. Double mutants that were constructed by transduction carried either sfx-1, sfx-2, or sfx-3 and one of exoA31, exoB13, or exoF55 in a Δ5408 of ΔF114 background. These constructs were then tested for sensitivity to deoxycholate, fluorescence on calcofluor media, and on alfalfa for suppression of the symbiotic phenotype associated with these exopolysaccharide mutants (Table 19). The results show that in the Δ5408/ΔF114

background that these strains were constructed in, the symbiotic phenotype associated with these exopolysaccharide mutations is not suppressed nor do they acquire the ability to synthesize calcofluor binding succinoglycan. They do, however, appear to affect the sensitivity of the bacteria to deoxycholate. The results suggest that loss of either exoB or exoF but not exoA in RmF346 or RmG425 reverses the sensitivity of these strains to deoxycholate. It is not clear how these loci interact at present but it does appear that sensitivity to deoxycholate in RmF346 and RmG425 requires loci that are also involved in succinoglycan biosynthesis in Rm1021.

## SUMMARY

This work was originally pursued with the idea that further characterization of the second site revertants may shed light on the role of fix114. It has been found that fix114 codes for membrane bound or secreted protein(s) and that fix114 mutations are blocked early in symbiosis. In this work evidence is presented that there are at least two distinct classes of second site revertants. One of these classes, Class II, appears to be sensitive to detergents that are associated with alterations in the outer cell envelope while the other class, Class I, is not to be sensitive to these same compounds. However, inserts which

map to within 20 kb of the Class I mutation sfx-1, exhibit the same phenotype as Class II revertants to these same detergents. It was also found that both classes of second site revertants suppress a conditional mucoid phenotype and that the detergent sensitivity of class II revertants can be reversed by a cosmid that carries a locus, exoZ, or by the introduction of exoB or exoF mutations which are involved in succinoglycan (EPS) synthesis. All this taken together suggests that an alteration in the cell surface has occurred in the second site revertants that allows strains which carry fix114 deletions to by-pass the original symbiotic block with alfalfa. These same mutations, however, are host specific since they do not completely restore symbiosis on sweet clover.

# APPENDIX 1

## CONSTRUCTION OF RmG591

RmG591 contains the sfx-1 allele in a Rm1021 background with no Tn5 or Tn5 derivatives present so that Tn5 inserts generated in pTH56 or another plasmid carrying the sfx-1 allele could be homogenotized into the chromosome. It was known that the sfx-1 allele was 20% linked in transduction with Ω5117::Tn5-233 in R. melilolti strain RmF994 and that pck-1::TnV was loosely linked by transduction (8 %) with Ω5117::Tn5-233. By converting transductional linkage to approximate kilobase distances (Wu 1966), pck-1::TnV in Rm5439 and sfx-1 in RmF994 should be seperated by approximately 160 kb. Since the packaging capacity of ΦM12 is approximately 150 kb (Finan et al. 1984) it is unlikely that sfx-1 and pck-1 are cotransducible. Therefore the following strategy was employed; a lysate of Rm5439 containing pck-1::TnV was transduced into RmF994 containig Ω5117::Tn5-233. $Nm^r$ transductants were screened for loss of $Gm^r$-$Sp^r$ and for cotransduction of the inability to grow on succinate as a sole carbon source. This isolate was single colony purified three times. Into this strain a wild type lysate was transduced and transductants were selected for their ability to grow on succinate as a sole carbon source. The resultant isolate was sensitive to Nm, Gm, Sp, resistant

to Sm and was able to grow on defined media with either glucose or succinate as a sole carbon source.

# APPENDIX 2

## SYMBIOTIC PHENOTYPES OF TnphoA INSERTS IN sfx-1 REGION

| Strain | Relevant Characteristics | Dry Wt. mg/plant | % Wild Type |
|---|---|---|---|
| Rm1021 | Wild type | 15.3 ± 0.4 | 100 |
| RmG624 | Ω9A::TnphoA | 13.5 ± 1.7 | 88 |
| RmG626 | Ω13A::TnphoA | 4.9 ± 0.4 | 32 |
| RmG627 | Ω20B::TnphoA | 11.9 ± 1.1 | 78 |
| RmG628 | Ω21A::TnphoA | 12.4 ± 1.1 | 81 |
| U. I.[a] | | 4.6 ± 0.1 | 30 |

Active TnphoA inserts that were isolated from pTH56 were homogenotized into RmG591 and inoculated onto alfalfa. Plants were harvested after 23 days and the shoots were dryed. Data is presented as the mean of three replicates ± standard error. Each replicate consisted of between 7-9 plants.

[a] uninoculated control

## LITERATURE CITED

**Abe, M., A. Amemura, S. Higashi.** 1982. Studies on $\beta$-1-2 glucan obtained from the periplasmic space of Rhizobium trifolii cells. Plant Soil **64:** 315-324.

**Ames, P., S. A. Schluendeberg, and K. Bergman.** 1980. Behavioural mutants of Rhizobium meliloti. J. Bacteriol. **141:** 722-727.

**Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.

**Baur, W. D.** 1981. Infection of legumes by Rhizobia. Ann. Rev. Plant Physiol. **32:** 407-449

**Berg, D. E., and C. M. Berg.** 1987. The prokaryotic transposon Tn5. Biol. Technol. **1:** 417-435.

**Beringer, J. E.** 1974. R-factor transfer in Rhizobium leguminosarum. J. Gen. Microbiol. **84:** 188-198.

**Beringer, J. E., J. L. Beynon, A. V. Buchanan-Wollaston, and A. W. B. Johnston.** 1978. Transfer of the drug resistance transposon Tn5 to Rhizobium. Nature **276:** 633-634.

**Bohlool, B. B., E. L. Schmidt.** 1974. Lectins: A possible basis for specificity in the Rhizobium legume nodule symbiosis. Science **185:** 269-271.

**Borthakur, D., J. A. Downie, A. W. B. Johnston and J. W. Lamb.** 1985. psi, a plasmid-linked Rhizobium phaseoli gene that inhibits polysaccharide production and which is required for symbiotic nitrogen fixation. Mol. Gen. Genet. **200:** 278-282.

**Borthakur, D., C. E. Barber, J. W. Lamb, M. J. Daniels, J. A. Downie, A. W. B. Johnston.** 1986. A mutation that blocks exopolysaccharide synthesis prevents nodulation of peas by R. phaseoli and is corrected by DNA from Rhizobium or the phytopathogen Xanthomonas. Mol. Gen. Genet. **203:** 320-323.

**Borthakur, D., and A. W. B. Johnston.** 1987. Sequence of psi, a gene on the symbiotic plasmid of Rhizobium phaseoli which inhibits exopolysaccharide synthesis and nodulation

and demonstration that its transcription is inhibited by psr, another gene on the symbiotic plasmid. Mol. Gen. Genet. **207:** 149-154.

**Bradely, D. J., G. W. Butcher, G. Galfre, E. A. Wood.** 1986. Physical association between the peribacteroid membrane and lipopolysaccharide from the bacteroid outer membrane in Rhizobium infected peas root nodules. J. Cell Sci. **85:** 47-61.

**Brewin, N. J., A. E. Wood, A. P. Larkins, G. Galfre, G. W. Butcher.** 1986. Analysis of lipopolysaccharide from root nodule bacteroids of Rhizobium leguminosarum using monoclonal antibodies. J. Gen. Microbiol. **132:** 1959-1968.

**Buikema, W. J., W. W. Szeto, P. V. Lemley, W. H. Orme-Johnson, and F. M. Ausubel.** 1985. Nitrogen fixation specific regulatory genes of Klebsiella pneumoniae and Rhizobium meliloti share homology with general nitrogen regulatory gene ntrC of K. pneumoniae nifB genes. Nucl. Acids Res. **13:** 4539-4555.

**Buikema, W. J., J. A. Klingensmith, S. L. Gibbons, and F. M. Ausubel.** 1987. Conservation of structure and location of Rhizobium meliloti and Klebsiella pneumoniae nifB genes. J. Bacteriol. **169:** 1120-1126.

**Carlson, R. W., R. Lee.** 1983. A comparison of the surface polysaccharide from Rhizobium leguminosarum 128C53 sm(r) rif(r) with the surface polysaccharides from its exo-1 mutant. Plant Physiol. **71:** 223-228.

**Carlson, R. W.** 1984. Heterogeneity of Rhiozobium lipopolysaccharides. J. Bacteriol. **158:** 1012-1017.

**Carlson, R. W., S. Kalembasa, D. Turowski, P. Pachori, K. D. Noel.** 1987. Characterization of the lipopolysaccharide from Rhizobium phaseoli mutant that is defective in infection thread development. J. Bacteriol. **169:** 4923-4928.

**Cava, J. R., T. Hong, K. D. Noel.** 1990. Mapping of complementation groups within a Rhizobium leguminosarum DFn42 chromosomal region required for lipopolysaccharide synthesis. Mol. Gen. Genet. **221:** 125-128.

**Chakravorty, A. K., W. Zurkowski, J. Shine, and B. J. Rolfe.** 1982. Symbiotic nitrogen fixation: Molecular cloning of Rhizobium genes involved in exopolysaccharide synthesis and effective nodulation. Mol. Gen. Genet. **192:** 459-465.

**Charles, T. C., T. M. Finan. 1991.** Analysis of a 1600 kilobase Rhizobium meliloti megaplasmid using defined deletions generated in vivo. Genetics in press

**Charles, T. C., T. M. Finan. 1990.** Genetic map of Rhizobium meliloti pRmeSU47b. J. Bacteriol. **172:** 2469-2476.

**Chen, H., M. Bately, J. Redmond, B. J. Rolfe.** 1985. Alteration of the effective nodulation of fast growing broad host range Rhizobium due to changes in exopolysaccharide synthesis. J. Plant Physiol. **120:** 331-349.

**Clover, R. H., J. Keiber, E. R. Signer.** 1989. Lipopolysaccharide mutants of Rhizobium meliloti are not defective in symbiosis. J. Bacteriol. **171:** 3961-3967.

**David, M., M. L. Daveram, 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.

**Dazzo, F. B. and G. L. Truchet.** 1983. Interactions of lectins and their saccharide receptors in Rhizobium-Legume symbiosis. J. Membrane Biol. **73:** 1-16.

**Deballe, F., C. Rosenberg, J. Vasse, F. Maillet, E. Martinez, J. Denaire, and G Truchet.** 1986. Assignment of symbiotic developmental phenotypes to common and specific nodulation (nod) genetic loci of Rhizobium meliloti. J. Bacteriol. **168:** 1075-1086.

**DeMaagd, R. A., S. R. Anuradha, I. H. G. M. Mulders, L. Goosen-deRoo, M. C. M. Vanloosdrect, C. A. Wijffelman, B. J. J. Lugtenberg.** 1989. Isolation and characterization of mutants of Rhizobium leguminosarum biovar viciae 248 with altered lipopolysaccharides: Possible role of surface charge or hydrophobicity in bacterial release from the infection thread. J. Bacteriol. **171:** 1143-1150.

**DeVos, G. F., G. Walker, and E. R. Signer.** 1986. Genetic manipulations in Rhizobium meliloti utilizing two new transposon Tn5 derivatives. Mol. Gen. Genet. **204:** 485-491.

**Diaz, C. L., L. S. Melchers, P. J. J. Hooykaas, B. J. J. Lugtenberg, J. W. Kijne.** 1989. Root lectin as a determinant of host-plant specificity in Rhizobium-legume symbiosis. Nature **388:** 579-581.

**Diebold, R., K. D. Noel.** 1989. Rhizobium leguminosarum exopolysaccharide mutants: Biochemical and genetic analysis and symbiotic behaviour on three hosts. J. Bacteriol. **171:** 4821-4830.

**Doherty, D., J. A. Leigh, J. Glazebrook, G. C. Walker.** Rhizobium meliloti mutants that overproduce the Rhizobium meliloti acidic calcofluor binding exopolysaccharide. J. Bacteriol. **170:** 4249-4256.

**Douglas, C. J., W. Halperin, E. W. Nester.** 1982. Agrobacterium tumefaciens mutants affected in attachment to plant cells. J. Bacteriol. **152:** 1265-1275.

**Dylan, T., L. Ielpi, S. Stanfield, L. Kashyap, C. Douglas, M. Yanofsky, E. Nester, D. R. Helinski, G. Ditta.** 1986. Rhizobium meliloti genes required for nodule development are related to chromosomal virulence genes in Agrobacterium tumefaciens. Proc. Natl. Acad. Sci. USA. **83:** 4403-4407.

**Dylan, T., D. R. Helinski, G. R. Ditta.** 1990a. Hypoosmotic adaptation in Rhizobium meliloti requires $\beta$-(1-2) glucan. J. Bacteriol. **172:** 1400-1408.

**Dylan, T., P. Nagpul, D. R. Helinski, G. R. Ditta.** 1990b. Symbiotic pseudorevertants of Rhizobium meliloti ndv mutants. J. Bacteriol. **172:** 1409-1417.

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

**Figurski, D. H., and D. R. Helinski.** Replication of an origin-containing derivative plasmid of RK2 dependant on a function provided in trans. Proc. Natl. Acad. Sci. USA **76:** 1648-1652.

**Finan, T. M., J. M. Wood, D. C. Jordan.** 1983. Symbiotic properties of $C_4$-dicarboxylic acid transport mutants of Rhizobium leguminosarum. J. Bacteriol. **154:** 1403-1423.

**Finan, T. M., E. Hartwieg, K. LeMieux, K. Bergman, G. C. Walker, and E. R. Signer.** 1984. General transduction in Rhizobium meliloti. J. Bacteriol. **159:** 120-124.

**Finan, T. M., A. M. Hirsch, J. A. Leigh, E. Johansen, G. A. Kuldau, S. Deegan, G. C. Walker, E. R. Signer.** 1985. Symbiotic mutants of Rhizobium meliloti that uncouple plant from bacterial differentiation. Cell **40:** 869-877.

**Finan, T. M., B. Kunkel, G. F. DeVos, E. R. Signer.** 1986. Second symbiotic megaplasmid carrying exopolysaccharide and thiamine biosynthetic genes. J. Bacteriol. **167:** 66-72.

**Finan, T. M., I. J. Oresnik, A. Bottacin.** 1988. Mutants of Rhizobium meliloti defective in succinate metabolism. J. Bacteriol. **170:** 3396-3403.

**Firmin, J. L., K. E. Wilson, L. Rossen, A. W. B. Johnston.** 1986. Flavonoid activation of nodulation genes in Rhizobium reversed by other compounds present in plants. Nature **324:** 90-92.

**Forrai, T., E. Vincze, Z. Banfalvi, G. B. Kiss, G. S. Randhava, A. Kondorosi.** 1983. Localization of symbiotic mutations in Rhizobium meliloti. J. Bacteriol. **153:** 635-643.

**Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of Rhizobium mutants. Gene **18:** 289-296.

**Furichi, T., M. Inouye, and S. Inouye.** 1985. Novel one-step cloning vector with a transposable element: application to the Myxococcus xanthus genome. J. Bacteriol. **164:** 270-275.

**Geremia, R. A., S. Cavaignac, A. Zorreguieta, N. Toro, J. Olivares, R. A. Ugalde.** 1987. A Rhizobium meliloti mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharides but fails to form $\beta$-(1-2) glucan. J. Bacteriol. **169:** 880-884.

**Glazebrook, J., G. C. Walker.** 1989. A novel exopolysaccharide can function in place of the calcofluor binding exopolysaccharide of Rhizobium meliloti. Cell **56:** 661-672.

**Hancock, R. E. W., and P. Reeves.** 1976. Lipolysaccharide deficient, bacteriophage resistant mutants of Escherichia coli K-12. J. Bacteriol. **127:** 98-108.

**Higashi, Y., J. L. Strominger, and C. C. Sweely.** 1967. Structure of a lipid intermediate in cell wall peptidoglycan synthesis: A derivative of a $C_{55}$ isoprenoid alcohol. Proc. Natl. Acad. Sci. USA. **57:** 1878-1884.

**Hohn, B.** 1979. In vitro packaging of λ and cosmid DNA. Methods Enzymol. **68:** 299-303.

**Hong, G. F., J. E. Burn, A. W. B. Johnston.** 1987. Evidence that DNA involved in the expression of nodulation (nod) genes in Rhizobium binds to the regulatory gene nodD. Nucl. Acids Res. **15:** 9677-9690.

**Horvath, B, C. W, Bachem, J. Scmidt, I. Torok, Z. Gyorgypal, I. Barabas, U. Wieneke, J. Schell, and A. Kondorosi.** 1986. Organization, structure, and symbiotic function or Rhizobium meliloti nodulation genes determining host specificity for alfalfa. Cell **46:** 335-343.

**Hraback, E. M., M. R. Urbano, F. B. Dazzo.** 1981. Growth phase dependent immunodeterminants of Rhizobium trifolii lipopolysaccharide which bind trifolin A, a white clover lectin. J. Bacteriol. **148:** 697-711.

**Hynes, M. F., R. Simon, P. Muller, K. Niehaus, M. Labes, A. Puhler.** 1986. The two megaplasmids of Rhizobium meliloti are involved in the effective nodulation of alfalfa. Mol. Gen. Genet. **202:** 356-362.

**Ielpi, L., T. Dylan, G. S. Ditta, D. R. Helinski, S. Stanfield.** 1990. The ndvB locus of Rhizobium meliloti encodes a 319-kDa protein involved in the production of β-(1-2) glucan. J. Biol. Chem. **265:** 2843-2851.

**Jones, J. D. G., and N. Gutterson.** 1987. An effecient mobilizable cosmid vector, pRK7813, and its use in a method for rapid marker exchange in Pseudomonas flourescens strain HV37a. Gene **61:** 299-306.

**Kamberger, W.** An Ouchterlony double diffusion study on the interaction between legume lectins and Rhizobium cell surface antigens. Arch Microbiol. **121:** 83-90.

**Kannenberg, E. L., N. J. Brewin.** 1989. Expression of a cell surface antigen from Rhizobium leguminosarum 3841 is regulated by oxygen and pH. J. Bacteriol. **171** 4543-4548.

**Kato, G., Y. Maruyama, M. Nakumura.** 1979. Role of lectins and lipopolysaccharides in the recognition process of specific Rhizobium-legume symbiosis. Agric. Biol. Chem. **43:** 1085-1092.

**Klein, S..** 1987. Ph.D. thesis, Dept. of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts.

**Law, I. J., A. J. Tamamoto, A. J. Mort, W. D. Mort, W. D. Baur.** 1982. Nodulation of soybean by Rhizobium japonicum mutants with altered capsule synthesis. Planta **154:** 100-109.

**Leigh, J. A., E. R. Signer, G. C. Walker.** 1985. Exopolysaccharide deficient mutants of Rhizobium meliloti that form ineffective nodules. Proc. Natl. Acad. Sci. USA **82:** 6231-6234.

**Leigh, J. A., J. W. Reed, J. F. Hanks, A. M. Hirsch, G. C. Walker.** 1987. Rhizobium meliloti mutants that fail to succinylate their calcoflour binding exopolysaccharides are defective in nodule invasion. Cell **51:** 579-587.

**Leigh, J. A., C. C. Lee.** 1988. Characterization of polysaccharide of Rhizobium meliloti exo mutants that form ineffective nodules. J. Bacteriol. **170:** 3327-3332.

**Lerouge, P., P. Roche, C. Faucher, F. Maillet, G. Truchet, J. C. Prome, and J. Denaire.** 1990. Symbiotic host specificity of Rhizobium meliloti is determined by a sulphated and acylated glucosamine oligo saccharide signal Nature **344:** 781-784.

**Long, S., J. W. Reed, J. Himawan, G. C. Walker.** 1988a. Genetic analysis of a cluster of genes required for the synthesis of the calcofluor binding exopolysaccharide of Rhizobium meliloti. J. Bacteriol. **170:** 4239-4248.

**Long, S., S. McCune, G. C. Walker.** 1988b. Symbiotic loci of Rhizobium meliloti identified by random TnphoA mutagenesis. J. Bacteriol. **170:** 4257-4265.

**Long, S. R., W. J. Buikema, and F. M. Ausubel.** 1982. Cloning of Rhizobium meliloti nodulation genes by direct complementation of Nod$^-$ mutants. Nature (London) **298:**485-488.

**Long, S. R.** 1989. Rhizobium-Legume Nodulation: Life Together in the Underground. Cell **56:** 203-214.

**Maier, R., and W. Brill.** 1976. Ineffective and non-nodulating mutant strains of Rhizobium japonicum. J. Bacteriol. **127:** 763-769.

**Maier, R., and W. Brill.** 1978. Involvement of Rhizobium japonicum O-antigen in soybean nodulation. J. Bacteriol. **128:** 1295-1299.

**Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular Cloning. Cold Spring Harbour, New York.

**Manoil, C., and J. Beckwith.** TnphoA, a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:** 8129-8133.

**Martinez, E., D. Romero, and R. Palacios.** 1990. The Rhizobium Genome. CRC Plant Sci. **9:** 59-93.

**Meade, H. M., and E. R. Signer.** 1977. Genetic mapping of Rhizobium meliloti. Proc. Natl. Acad. Sci. USA. **74:** 2076-2078.

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

**Miller, J. H.** 1972. Experiments in molecular genetics. CSH Laboratory,Cold Spring Harbour, New York.

**Miller, K. J., E. P. Kennedy, V. N. Reinhold.** 1986. Osmotic adaptation by Gram negative bacteria: Possible role for periplasmic oligosaccharides. Science **231:** 48-51.

**Muller, P., M. Hynes, D. Kapp, K. Niehaus, A, Puhler.** 1988. Two classes of Rhizobium meliloti infection mutants differ in exopolysaccharide production and in co-inoculation properties with nodulation mutants. Mol. Gen. Genet. **211:** 17-26.

**Mulligan, J. T., and S. R. Long.** 1985. Induction of Rhizobium meliloti nodC expression by plant exudate requires nodD. Proc. Natl. Acad. Sci. USA **82:**6609-6613.

**Napoli, C., P. Alberseim.** 1980. Rhizobium leguminosarum mutants incapable of normal polysaccharide production. J. Bacteriol. **144:** 630-640.

**Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49:** 1-32.

**Noel, K. D., K. A. Vanden Bosch, B. Kulpaca.** 1986. Mutations in Rhizobium phaseoli that lead to arrested development of infection threads. J. Bacteriol. **168:** 1392-1401.

**Nutman, P. S.** 1952. Physiological studies on nodule formation.II. The influence of delayed inoculation on the rate of nodulation in red clover. Ann. Bot. **13:** 261-283.

**O'Connell, K. P., J. Handelsman.** 1989. chvA locus may be involved in export of neutral $\beta$-1,2-linked glucan from Agrobacterium tumefaciens. Molec. Plant Microbe Interact. **2:** 11-16.

**Peters, N. K., J. W. Frost, and S. R, Long.** 1986. A plant flavone, luteolin, induces expression of Rhizobium meliloti nodulation genes. Science **233:** 977-980.

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

**Putnoky, P., E. Grosskapf, D. T. Canatta, G. B. Kiss, A. Kondorosi.** 1988. Rhizobium fix genes mediate at least two communication steps in symbiotic nodule development. J. Cell Biol. **106:** 597-607.

**Putnoky, P., G. Petrovics, A. Kerszt, E. Groskopf, D. T. Cam Ha, Z. Banfalvi, and A. Kondorosi.** 1990. Rhizobium meliloti lipopolysaccharide and exopolysaccharide can have the same function in plant-bacterium interaction. J. Bacteriol. **172:** 5450-5458.

**Puvanesarajah, V. F., M. Schell, G. Stacey, C. J. Douglas, E. W. Nester.** 1985. A role for 2-linked $\beta$-D-glucans in the virulence of Agrobacterium tumefaciens. J. Bacteriol. **164:** 102-106.

**Ronson, C. W., P. Lyttleton, and J. G. Robertson.** 1981. $C_4$-dicarboxylate transport mutants of Rhizobium trifolii form ineffective nodules on Trifolium repens. Proc. Notl. Acad. Sci. USA. **78:** 4284-4288.

**Ronson, C. W., B. T. Nixon, L. M. Albright, F. M. Ausubel.** 1987. Rhizobium meliloti ntrA (rpoN) gene is reqired for diverse metabolic functions. J. Bacteriol. **169:** 2421-2431.

**Rostas, K., E. Kondorosi, B. Horvath, A. Simoncsits, and A. Kondorosi.** 1986. Conservation of extended promoter regions of nodulation genes in Rhizobium. Proc. Natl. Acad. Sci. USA **83:** 1757-1761.

**Ruvkin, G. B., and F. M. Ausubel.** 1981. A general method for site directed mutagenesis in prokaryotes. Nature **289:** 85-88.

**Sanderson, K. E., MacAlister and J. W. Costerton.** 1974. Permeability of lipopolysaccharide deficient (rough) mutants of Salmonella typhimurium to antibiotics, lysozyme, and other agents. Can. J. Microbiol. **20:** 1135-1145.

**Schweizer, M., H. Schwarz, I. Sonntag, and U. Henning.** 1976. Mutational change of membrane architecture. Mutants of Escherchia coli K-12 missing major proteins of the outer cell envelope. Biochim. Biophys. Acta **448:** 474-491.

**Siewert, G., J. L. Strominger.** 1967. Bacitracin: An inhibition of lipid pyrophosphate, an intermediate in biosynthesis of the peptidoglycan of bacterial cell walls. Proc. Natl. Acad. Sci. USA. **57:** 767-773.

**Simon, R.** 1984. High frequency mobilization of gram negative bacterial replicons by the in vitro constructed Tn5-mob transposon. Mol. Gen. Genet. **196:** 413-420.

**Stanfield, S. W., L. Ielpi, D. O'Brochta, D. R. Helinski, G. S. Ditta.** 1988. The ndvA gene product of Rhizobium meliloti is required for $\beta$-(1-2) glucan production and has homology to the ATP binding export protein HylB. J. Bacteriol. **170:** 3523-3530.

**Stanley, J., D. N. Dowling, M. Stucker, and W. J. Broughton.** 1987. Screening costramid libraries for chromosomal genes: An alternative interspecefic hybridization method. FEMS Microbiol. Lett. **48:** 25-30.

**Stone, K. J. and J. L. Strominger.** 1971. Mechanism of action of bacitracin: Complexation with metal ion and $C_{55}$ isoprenyl pyrophosphate. Proc. Natl. Acad. Sci. USA. **68:** 3223-3227.

**Sukupolvi, S., M. Vaara, I. M. Helander, P. Viljanen, and P. H. Makela.** 1984. New Salmonella typhimurium mutants with altered outer membrane permeability. J. Bacteriol. **159:** 704-712.

**Southern, E. M..** 1975. Detection of specific sequences among DNA fragments seperated by gel electrophoresis. J. Mol. Biol. **98:** 503-517.

**Sutton, W. D., A. D. Patterson.** 1980. Effects of the plant host on the detergent sensitivity and viability of Rhizobium bacteroids. Planta **148:** 287-292.

**Vanden Bosch, K. A., N. J. Brewin, E. L. Kannenberg.** 1989. Developmental regulation of Rhizobium cell surface antigen during growth of pea nodules. J. Bacteriol. **171:** 4537-4542.

**Vincent, J. M.** 1980. A manual for the practical study of root nodule bacteria. International Biological Programme hand book no. 15. Blackwell Scientific Publications, Oxford.

**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 located on a symbiotic megaplasmid. J. Bacteriol. **170:** 927-934.

**Williams, M. N. V., S. Klein, and E. R. Signer.** 1989. Host restriction and transduction in Rhizobium meliloti. Appl. Environ. Microbiol. **55:** 3229-3223.

**Williams, M. N. V., R. I. Hollingsworth, S. Klein, E. R. Signer.** 1990. The symbiotic defect of Rhizobium meliloti exopolysaccharide mutant is suppressed by lpsZ$^+$, a gene involved in lipopolysaccharide biosynthesis. J. Bacteriol. **172:** 2622-2632.

**Wood, E. A., G. W. Butcher, N. J. Brewin.** 1989. Genetic derepression of a developmentally regulated lipopolysaccharide antigen from Rhizobium leguminosarum 3841. J. Bacteriol. **171:** 4549-4555.

**Wright, A., M. Dankert, P. Fennessey, and P. W. Robbins.** 1967. Characterization of a polyisoprenoid compound functional in O antigen biosynthesis. Proc. Natl. Acad. Sci. USA. **57:** 1798-1803.

**Wu, T. T.** 1966. A model for three-point analysis of random general transduction. Genetics **54:** 405-410.

**Yanish-Perron, C., J. Viera, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:** 103-119.

**Yarosh, O. K., T. C. Charles, and T. M. Finan.** 1989. Analysis of $C_4$-dicarboxylate transport genes in Rhizobium meliloti. Mol. Micobiol. **3:** 813-823.

**York, W. S., M. McNeil, A. G. Darvill, P. Albersheim.** 1980. Beta-2 linked glucans secreted by fast growing species of Rhizobium. J. Bacteriol. **142:** 243-248.

**Zahn, H., S. B. Levery. C. C. Lee, J. A. Leigh.** 1989. A second exopolysaccharide of Rhizobium meliloti strain SU47 that can function in root nodule invasion. Proc. Natl. Acad. Sci. USA. **86:** 3055-3059.

**Zevenhuizen, L. P. T. M., H. J. Scholten-Koerselman.** 1979. Surface carbohydrates of Rhizobium.I. $\beta$ 1-2 glucans. Antonie van Leeuwenhook J. Microbiol. **45:** 165-175.

**Zevenhuizen, L. P. T. M., I. Scholten-Koerselman, M. A. Tosthumus.** 1980. Lipopolysaccharides of Rhizobium. Arch. Microbiol. **125:** 1-8.

**Zorreguieta, A, R. A. Ugalde.** 1986. Formation in Rhizobium and Agrobacterium species of a 235 kilodalton protein intermediate in $\beta$-(1-2) glucan synthesis. J. Bacteriol. **167:** 947-951.