THE ROLE OF *RHIZOBIUM MELILOTI* EXOPOLYSACCHARIDE IN NODULATION OF ALFALFA

by

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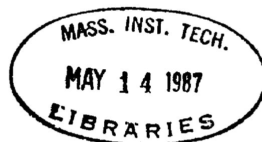

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ABSTRACT

Among the genes of *Rhizobium meliloti* that affect nitrogen-fixing symbiosis with alfalfa are *nod* genes, in which mutations block or delay formation of nitrogen-fixing root nodules, and *exo* genes, in which mutations allow nodule formation but block nodule invasion, nitrogen fixation and production of a rhizobial exopolysaccharide which binds the fluorescent dye calcofluor-white. The role of *exo* genes and their products in nodulation is examined.

Nodule induction by *exo* mutants apparently occurs by intercellular rather than root-hair-mediated, intracellular invasion. The functions of all the known *nod* genes are shown to be required for nodule induction by *exo* mutants, as *nod exo* double mutants have the same nodulation phenotype as single *nod* mutants.

An interaction of Nod and Exo functions is deduced from a series of experiments involving coinoculation of various mutants. The ability of one coinoculant to "help" (i.e. reverse the symbiotic defect of) the other for Exo depends on its *nod* genotype. The interaction of Nod and Exo affects nodule invasion and, directly or indirectly, bacteroid differentiation.

Mutations at several *exo* loci in a natural isolate of *R. meliloti*, Rm41, do not block production of nitrogen-fixing nodules although they do block production of the calcofluor-white-binding exopolysaccharide. Mutants at the same loci in *R. meliloti* SU47 background induce nodules which cannot fix nitrogen. The nature of the natural "suppressor" of the Fix$^-$ phenotype of *exo* mutants in the Rm41 background is not known.

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This study would never have been completed were I not confident that my children, Avraham Yosef and Ezra Yoel, were in the best of hands while I was at the lab. My heartfelt appreciation is due to Sara and Zeev Hollender and to the entire staff of Yaldaynew Daycare Center.

Many friends and relatives have given us moral support over the years, none more unstintingly than my very dear parents-in-law. This thesis is dedicated to my parents, who set me on this path, and to my husband, Yitzhak, who alone knows how much of his time, effort and will are bound in my career.

For my parents

who dared me

and for Yitzhak

who also took up the dare

Rabbi Tarfon says: The day is short, there is much work, the workers are lazy, the reward is large, and the landlord is knocking...
He was wont to say: It is not incumbent upon you to finish the work, but neither are you free to desist from it...

-Mishnah Avot 2, 20-21

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CHAPTER 1: INTRODUCTION

A BRIEF SURVEY OF THE GENETICS OF ALFALFA NODULATION BY *Rhizobium meliloti*

The *Rhizobium*-legume symbiosis is well-known: rhizobia are soil bacteria which can infect the roots of leguminous plants as well as some non-legumes, inducing the formation of root nodules. Within these nodules, the bacteria differentiate into nitrogen-fixing bacteroids, supplying fixed nitrogen to the plants in exchange for photosynthate. The processes leading to the establishment and maintenance of an effective symbiosis are complex, requiring bacterial and plant factors. The symbiosis has obvious significance to agriculture and ecology, and understanding the molecular processes involved will probably also lead to a better understanding of bacterial-plant interactions in general.

In this introduction, I will deal only with those aspects of nodulation which are pertinent to my thesis. I will not discuss nitrogen fixation, per se, or the role of the plant host in this very complex interaction, but will restrict this discussion to brief descriptions of nodule development and of the bacterial genes which are relevant to this study. Several extensive reviews are referenced (Dart, 1977; Bauer, 1981; Newcomb, 1981; Verma and Long, 1983; Long, 1984: Halverson and Stacey, 1986).

Specificity of the *Rhizobium*-Legume Interaction: The interaction of particular rhizobia with particular legumes tends to be selective, each *Rhizobium* species nodulating specific groups of related legumes. The

most common cross-inoculation groups are as follows: *R. meliloti* -- *Medicago* (alfalfa), *Melilotus*, *Trigonella*; *R. trifolii* -- *Trifolium* (clover); *R. leguminosarum* -- *Pisum* (pea), *Vicia*, *Lathyrus*; *R. phaseoli* -- *Phaseolus* (bean); *R. lupini* -- *Lupinus*; *R. japonicum* -- *Glycine* (soybean), some cowpea; and *Rhizobium sp.* -- *Vigna* (cowpea). *R. parasponia* nodulate the non-leguminous *Parasponia*, as well as some members of the cowpea group (Allen and Allen, 1981; Long, 1984; Dart, 1977). Some of the *R. japonicum* groups and cowpea rhizobia have been reclassified as *Bradyrhizobium*, a genus comprised of the slow-growing rhizobia. *R. trifolii*, *R. leguminosarum* and *R. phaseoli* are all fast-growers and appear to be closely related, based on DNA homology and marker rescue experiments. *R. meliloti*, also a fast-grower, is slightly less closely related to *R. leguminosarum* and slightly more closely related to *Agrobacterium tumefaciens* (Long, 1984). The relatedness of different rhizobial species to each other and to *A. tumefaciens* (also a member of the family *Rhizobiaceae*) is important when interpreting experiments involving transfer of symbiotic properties from one species to another.

Nodule morphogenesis: The stages in nodule development can be summarized as follows (Long, 1984; after Vincent, 1974; 1980): root colonization (Roc); attachment to root (Roa); root hair curling (Hac); infection thread formation (Inf); nodule initiation (Noi); bacterial release (Bar); bacteroid differentiation (Bad); nitrogen fixation (Nif); nodule persistence (function) (Nop).

Rhizobial attachment to the root hairs of homologous hosts results in characteristic deformation of the normally straight hairs. Wild type

R. meliloti induce root hair curling (Hac), which often results in tight 360° curls called "shepherd's crooks". A refractile "bright" or "hyaline spot" within the crook appears to be the site of initiation of the infection thread (Inf) down which the bacteria migrate into the nodule cortex. Studies on *R. trifolii* (Callaham and Torrey, 1981) and on *Rhizobium* sp. ANU240 (Ridge and Rolfe, 1985) indicate localized degradation of the host cell wall at the site of infection. The infection thread wall is continuous with the new host cell wall which is resynthesized at the point of infection.

The infection thread grows toward the root cortex, crossing cell wall boundaries, as the bacteria within multiply. Sometimes, the infection thread continues growth intercellularly, proceeding through the middle lamella between plant cell walls. Infection threads branch frequently. Within the thread the bacteria are surrounded by a matrix which may be of bacterial origin. Bacteria are released (Bar) in membrane-enclosed "unwalled droplets" from unwalled regions of the infection thread (Newcomb, 1981).

As the bacteria invade, the root cells divide. The first mitoses of root inner cortex occur as early as 21 hours post-inoculation (Dudley and Long, 1985). Nodules of alfalfa are "indeterminate", that is, they grow in length from a defined meristem, producing cylindrical nodules. Other species form "determinate" nodules which are spherical.

Behind the meristem is the bacteroid zone, containing the infected cells. The cells in the bacteroid region are enlarged and packed densely with bacteroids, each of which is surrounded by a peribacteroid membrane derived from the host plasma membrane. The bacteroids differentiate to the nitrogen-fixing forms which are enlarged and may be

Y-shaped (although *R. meliloti* bacteroids usually do not branch). Behind the bacteroid zone lies a region of senescence, where host and bacterial cells degenerate. (For review of nodule morphogenesis see Newcomb, 1981.)

A number of plant-derived nodule-specific proteins ("nodulins") have been identified, including leghaemoglobin. This protein regulates delivery of oxygen to the oxygen-sensitive nitrogenase complex, the bacterial enzyme which reduces nitrogen to ammonia.

Genetics of *Rhizobium*: In this study, I make extensive use of two principal means of introducing DNA into *R. meliloti*: conjugation, using various plasmids discussed below, and transduction, using rhizobiophage ϕM12 (Finan et al., 1984). Although transformation of *R. meliloti* has been reported (Selvaraj and Iyer, 1981), we have never obtained efficient transformation in our laboratory.

A number of broad host-range plasmids have been developed for use in *Rhizobium* (e.g. Ditta et al., 1980; Priefer et al., 1985; Tait et al.,1983). The most successful are derived from RP4, a P-group plasmid. In order to limit vector size, a triparental mating system is frequently used, whereby mobilization factors are provided in *trans* by a plasmid which cannot itself replicate in *Rhizobium*. Such a system was employed to complement *nod* mutants of *R. meliloti* by Long et al. (1982) who isolated a 20 kb region of DNA encoding functions required for nodulation, and is now used routinely for introduction of markers from recombinant DNA libraries.

Insertion mutagenesis of *R. meliloti* DNA can be accomplished by introduction of a mobilizable plasmid which cannot replicate in

Rhizobium, followed by selection for rescue of a drug marker on the insert. A number of such "suicide vectors" exist (Beringer et al., 1978; Figurski and Helinski, 1979; Simon et al., 1983). The only transposon which has been shown to transpose randomly in *R. meliloti* is Tn*5*, which encodes resistance to neomycin, bleomycin and streptomycin, but several derivatives of Tn*5* exist which carry different antibiotic markers (e.g. Avery and Kaiser, 1983; De Vos et al., 1986). Mutations constructed *in vitro* are generally transformed into *Eschericia coli* and mobilized into *R. meliloti* where they can be homogenotized by homologous recombination with genomic DNA (Ruvkun and Ausubel, 1981). In such experiments, either the vector is a plasmid which cannot replicate in *R. meliloti* or homogenotization is selected for by introduction of a plasmid which is incompatible with the vector followed by selection for marker(s) on that incoming plasmid.

Plasmid RP4 itself (or its derivative R68.45) can mobilize segments of chromosome in an apparently random fashion and this property has been used in developing genetic maps of *Rhizobium* (Meade and Signer, 1977; Kondorosi et al., 1977; Kondorosi et al., 1980). Such R-prime plasmids have also been used to localize specific genetic markers (e.g..Juillot et. al., 1984). The origin of transfer of RP4 has been cloned into transposon Tn*5* (Tn*5*-*Mob*; Simon, 1984; Tn*5*-*oriT*; Yakobson and Guiney, 1984). We have used Tn*5*-*Mob* for directed mobilization of *R. meliloti* chromosomal segments in mapping studies (see Appendix). Similarly, Tn*5*-*oriT* has been used for mobilization of symbiotic "megaplasmids" (Finan et al., 1986).

Fast-growing rhizobia characteristically have large megaplasmids. These have frequently been demonstrated to encode symbiotic functions

(Denarie et al., 1981). Some of these megaplasmids are self-transmissible, but not those of *R. meliloti*. Transfer of such plasmids to mutants has been shown to restore Nod$^+$ or Fix$^+$ phenotype (Djordjevic et al., 1983) and transfer of megaplasmids from given rhizobial species into other species extended the host range of the recipients (Hooykaas et al., 1981).

Strains of *R. meliloti* have various numbers of these large plasmids. For instance, *R. meliloti* SU47 has at least two symbiotic megaplasmids: pRmeSU47a includes genes required for the initial steps in nodulation (*nod*) and genes required for nitrogen fixation per se, *nif* and *fix*. (*Nif* genes were isolated by DNA sequence homology with the free-living nitrogen fixer *Klebsiella pneumoniae*; *fix* loci were identified in *Rhizobium*.) A second megaplasmid, pRmeSU47b, has some of the loci required for exopolysaccharide production (Finan et al., 1986). *R. meliloti* strain 41 harbours at least three large plasmids: pRme41a (about 200 kb); pRme41b (carries *nod-nif* region; about 1500 kb); and pRme41c (about 1500 kb; Banfalvi et al., 1985). There is evidence for surface exclusion of homologous megaplasmids among *R. meliloti* strains (ibid.).

Nod genes: In *R. meliloti*, a number of genes have been shown to be required for nodule initiation. These are located in two clusters spanning a 16 kb region of one of the symbiotic megaplasmids (pSymA=pRmeSU47a for *R. meliloti* SU47; pRme41b for *R. meliloti* 41). One of these clusters includes the so-called "common *nod*" genes, *nodDABC* (Kondorosi et al., 1984). Mutations in these genes can be complemented by homologous genes from other fast-growing rhizobia, namely, *R.*

leguminosarum and *R. trifolii*. Mutations in *nodA*, *nodB* or *nodC* abolish root hair curling (Hac$^-$), which is the earliest visible stage of nodulation. Such mutants are Nod$^-$, i. e. they fail to induce nodule-formation. In *R. meliloti*, the *nodA*, *B* and *C* genes are in one transcriptional unit, and are activated by addition of luteolin, which is present in alfalfa exudates (Peters et al., 1986). Induction of *nodABC* is dependent upon *nodD*, which is transcribed divergently from *nodABC* and is expressed constitutively (Mulligan and Long, 1985; Egelhoff and Long, 1985). The product of the *nodC* gene is membrane-associated (John et al., 1985; Schmidt et al., 1986). There are three homologues of *nodD* in *R. meliloti*, which appear to be required to different extents for nodulation of *Medicago* and *Melilotus* (Honma and Ausubel, 1986).

The second cluster includes four genes required for host-specificity of nodulation, *nodGEFH*. *R. meliloti* mutants fail to induce nodules (*nodH*) or show delayed nodulation (*nodG,E* or *F*) on the homologous host, alfalfa. In addition, they induce hair-curling or deformation on heterologous hosts which show no root hair response upon infection with wild type *R. meliloti*. Mutations in these genes cannot be complemented by homologous genes from other fast-growing *Rhizobium* species (Debelle et al., 1986; Horvath et al., 1986). Although the functions of these genes are not known, the predicted amino acid sequence of the *nodF* gene product has homology to acyl carrier proteins (Downie and Johnston, 1986).

In *R. leguminosarum* two additional genes, *nodI* and *nodJ*, are in the same transcriptional unit as *nodABC*. There is some evidence for sequence homologous to *nodI* in *R. meliloti* (Rossen et al., 1986). NodI

protein shares a consensus sequence with a diverse series of ATP-binding proteins and may serve to couple nodulation and ATP hydrolysis (Higgins et al., 1986).

Regulation of *nod* genes depends on a consensus sequence ("*nod* box") which has been found upstream of *nod* genes from *R. meliloti*, *R. leguminosarum* and *R. trifolii*. The integrity of this consensus sequence is required for nodule induction (Rostas et al., 1986). Although this regulation is likely to involve *nodD*, this has not yet been demonstrated. In *R. leguminosarum*, *nodD*-dependent induction by plant exudate of *nodFE* and *nodIJ* has been shown, as well as *nodABC* induction (Rossen et al., 1986). Similarly, induction by flavones of *nodABC* and *nodFE* of *R. trifolii* has been demonstrated (Redmond et al., 1986). The involvement of flavenoid compounds in activation of *nod* genes is apparently complex (Rossen et al., 1986).

Role of polysaccharides in nodulation: Surface polysaccharides produced by *Rhizobium* which have been identified include: extracellular polysaccharide (EPS), capsular polysaccharide (CPS), lipopolysaccharide (LPS), gel-forming polysaccharide and cyclic β-2-glucans (Dudman,1983). There is some ambiguity in the use of these terms but in general EPS refers to polysaccharide which is secreted into the medium, CPS to polysaccharide which remains bound to the exterior of cells after pelleting of cells, and LPS to lipid-containing polysaccharide which is at least partly embedded in the bacterial membrane. Although these surface molecules have long been speculated to have a role in nodulation, until recently the evidence for such a role was, at best, shaky.

<u>Exopolysaccharides (EPS):</u> Strains of *Rhizobium* produce acidic extracellular polysaccharides and the structures of some of these have been determined. There is a repeating oligosaccharide unit, the carbohydrate backbone of which may be neutral or acidic. Attached to this backbone are several side-chains, which generally include acidic substituents. Based on structural studies, it is unlikely that these polysaccharides are the primary determinants of host recognition: polysaccharides from strains which infect different hosts have nearly-identical structures, whereas some polysaccharides from strains infecting the same hosts have very different structures (Dudman, 1981; Bellogin et al., 1984; Bauer, 1981; McNeil et al., 1986).

An unequivocal role for EPS in nodulation was demonstrated by Leigh et al. (1985). The EPS of *R. meliloti* SU47 derivative Rm1021 had been previously characterized (Aman et al., 1981) and determined to have the structure shown in Fig. 1.1. Leigh et al. (1985) found that wild type Rm1021 bound a fluorescent dye, calcofluor-white, and isolated mutants (*exo*) which no longer bound that dye. All such mutants have a characteristic phenotype: they fail to produce the aforementioned acidic EPS and they induce the formation of nodules on alfalfa which are unable to fix nitrogen (Fix^-). The mutants fail to induce normal root-hair curling, or induce curling at lower frequency than wild type, sometimes causing regions of hypercurling. Nodules induced by *exoB* or *exoF* mutants lack infection threads; those induced by *exoA* mutants initiate infection threads late and development of these threads is aborted. The nodules are usually small, white or tan, but are sometimes large and tumorous-looking. Ultrastructurally, they have been shown to be devoid of differentiated bacteroids. Such bacteria as are found in

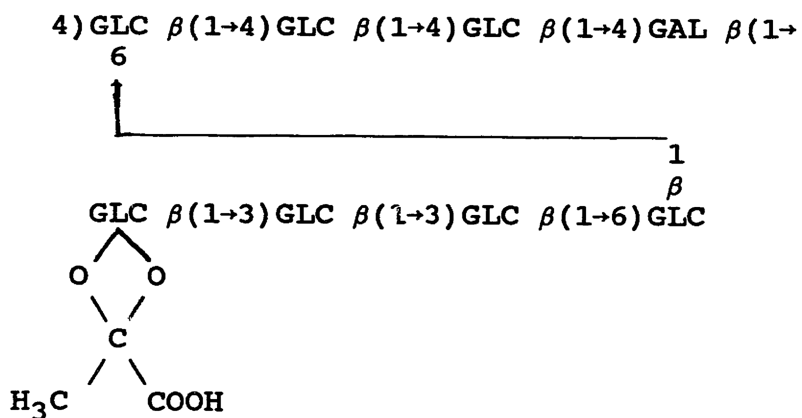

Fig.1.1. Reported structure of the *R. meliloti* calcofluor-binding acidic EPS (Leigh et al., 1985; Aman et al., 1981). *O*-succinyl and *O*-acetyl groups are also present at undetermined sites.

nodules induced by *exoB* or *exoF* mutants at least are restricted to intercellular spaces and have apparently entered the nodule intercellularly, rather than via infection threads (Finan et al., 1985; Leigh et al., 1985; A. M. Hirsch, pers. comm.). Mutants that might be similar to *R. meliloti exo* mutants have now been reported for *R. leguminosarum* (Borthakur et al., 1986) and *R. trifolii* (Djordjevic et al., 1987). EPS-deficient mutants of *R. phaseoli* have a Fix^+ phenotype (D. Noel, pers. comm.).

To date, some fifteen or so *exo* loci have been identified (Sue Long and Jason Reed, pers. comm.), including *exoH*, mutants of which produce altered EPS and also induce abortive infection thread formation and Fix^- nodules (Leigh et al., in preparation). It is not yet known which genes encode enzymes required for assembly of the polysaccharide structure and which are regulatory.

Strains of *R. meliloti* have been shown to produce more than one EPS (Yu et al., 1981; Yu et al., 1983; Dudman, 1981). A second mutation in some Rm1021*exo* derivatives activates expression of a cryptic EPS which apparently suppresses the Fix^- phenotype of those mutants (Jane Glazebrook, pers. comm.). The production of multiple EPS which can apparently substitute for one another in nodulation may be one reason why many earlier studies on EPS^- mutants were ambiguous (for review, see Dudman, 1984; Bauer, 1981).

R. meliloti has also been shown to secrete oligosaccharides which have the same structure as the repeating units of the polysaccharides (Zevenhuizen and van Neerven, 1983; Amemura et al., 1983). It is not known whether these are intermediates in polysaccharide synthesis or degradation products.

Other workers have reported effects of EPS in nodulation. The non-carbohydrate substitutions of *R. trifolii* polysaccharide have been correlated with inhibition of nodulation by extraneously added EPS (Skorupska et al., 1985). Chen et al. (1985) reported various phenotypic defects for different classes of EPS-deficient mutants of a broad host range *Rhizobium*, including formation of Fix$^-$ nodules and alteration in host range. Restoration of nitrogen-fixing ability of EPS-deficient mutants of broad host range *Rhizobium* and of *R. trifolii* by addition of exogenous EPS has been reported (Djordjevic et al., 1987).

<u>Regulation of polysaccharide synthesis:</u> In *R. phaseoli*, genes for polysaccharide regulation are located on the *nod-nif* megaplasmid. Mutations in a locus called *psi* (for polysaccharide inhibition) are Nod$^+$ Fix$^-$. When *psi* is introduced into *R. phaseoli* on a multicopy vector, polysaccharide production and nodulation are inhibited. Another gene, *psr* (polysaccharide restoration), inhibits transcription of *psi*. It has been suggested that these genes are involved in regulating bacteroid-specific versus free-living stage activation of symbiotic genes (Borthakur et al., 1985). Although a similar system has not been reported for *R. meliloti*, there is preliminary evidence for a locus (*nodM*) in *R. meliloti* which affects both nodulation and regulation of polysaccharide production (S. R. Long, pers. comm.).

Physiologically, secretion of EPS into culture medium can be increased by starvation of *R. meliloti* for nitrogen, sulphur or phosphorus (Leigh et al., 1985 and pers. comm.). Instead of starving cells in nitrogen-free medium, cultures can be grown in medium

containing glutamate (Dudman, 1964). Buildup of acid in starvation buffer apparently inhibits EPS synthesis and this can be avoided by choice of suitable carbon source, such as mannitol (Courtois et al., 1979).

Other Cell Surface Polysaccharides: A role in nodulation for β-2-glucan is suggested by the following. *Agrobacterium tumefaciens chvB* mutants are avirulent, show reduced attachment to plant surfaces and fail to produce β-2-glucan. A region which is homologous to *chvB* was identified in several *Rhizobium* species (*ndvB*). *R. meliloti* derivatives with mutations in *ndvB* produce normal amounts of the calcofluor-binding EPS but induce the formation of Fix$^-$ nodules which are empty of bacteroids and lack infection threads. Root-hair curling is normal (Puvanesarajah et al., 1985; Dylan et al., 1986). Another group demonstrated the absence of β-2-glucan from an *R. meliloti* mutant which induced similar nodules on alfalfa. The mutation in this strain had not been mapped at the time of publication (Geremia et al., 1987).

Mutants of *R. meliloti* which have altered LPS also induce the formation of Fix$^-$ nodules on alfalfa (Ralph Clover, et al.). These nodules do have infection threads, but development of these infection threads is aborted early. Development is arrested at a later stage than for some of the *exo* mutants which have no infection threads (A. M. Hirsch, pers. comm.). More nodulins (i.e. nodule-specific proteins of plant origin) are detected from these nodules than from nodules induced by *exo* mutants (Joanna Hanks et al., pers. comm.). Similar mutants have been reported for *R. phaseoli* (Noel et al., 1986). Furthermore, a mutant of *Bradyrhizobium japonicum* which lacks LPS fails to elicit

nodule formation (Puvanesarajah et al., 1987). Galactosyltransferase activity has been reported to affect bacteriophage receptors (possibly LPS?) as well as competitiveness of *R. meliloti* in nodulation (Ugalde et al., 1986).

Binding of *R. trifolii* to clover root hairs is believed to involve interaction between the plant lectin trifoliin A and bacterial capsular polysaccharide (Dazzo et al., 1985). The precise role of binding has been difficult to define because modifications of the CPS are apparently dependent on growth stage. Mutants with altered CPS induced more nodules than wild type on some species of clover and induced nodules with reduced nitrogen-fixing ability on another species of clover. The mutant CPS had increased levels of pyruvate substitutions (Gardiol et al., 1987).

It has long been believed that surface polysaccharides must play a role in infection of legumes by *Rhizobium* and evidence is now accumulating that deficiencies in a variety of surface polysaccharides are correlated with symbiotic deficiencies. In this study, we examine the role of *exo* genes and their products in nodulation of alfalfa by *R. meliloti*. Mutations in a variety of *exo* loci affect nodule development (Finan et al., 1985; Leigh et al., 1985; see above). The precise role of *exo* in symbiosis is not yet known but, as becomes clear in this thesis, its function is complex. In chapter 2, we demonstrate that the *nod* genes are required for intercellular nodulation by *exo* mutants, as *nod* is epistatic to *exo*. In chapter 3, Exo function is shown to be required for bacteroid differentiation (Bad) as well as for nodule

invasion (Noi). An interaction of Nod and Exo function is deduced from a series of experiments involving coinoculation of various mutants. Chapter 4 describes genetic manipulations which show that a natural "suppressor" which confers Fix^+ phenotype to *exo* mutants exists in a natural isolate of *R. meliloti*. The nature of the suppressor is not known. The thesis closes with a brief consideration of perspective.

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CHAPTER 2: ALL THE *NOD* GENES OF *RHIZOBIUM MELILOTI* ARE REQUIRED FOR INTERCELLULAR NODULATION BY *EXO* MUTANTS

INTRODUCTION

Rhizobium meliloti induce the formation of root nodules on alfalfa. For wild type *R. meliloti*, nodule formation begins with attachment of rhizobia to root hairs, which are induced to curl and form so-called shepherd's crooks. The bacteria enter the root hair within the curl and elicit the formation of an infection thread down which they travel across cell borders and into the root cortex. Upon release from the root hair, the bacteria differentiate into nitrogen-fixing bacteroids.

At least 10 *nod* genes have been identified which affect the early stages of nodule development (for overview see ref 6 and 18). In *R. meliloti* SU47, these genes are situated on a large symbiotic megaplasmid, pRmeSU47a. The "common *nod*" genes (*nodD,A,B,C*) affect root hair curling and infection thread formation. Mutations in these loci block nodule induction, but can be complemented by homologous genes from other *Rhizobium* species (e.g. 5, 16). In addition to the *nodD* locus in the "common *nod*" cluster (*nodD1*) two highly homologous copies are present in *R. meliloti* SU47 (*nodD2* and *nodD3*; M. Honma, pers. comm.). Other early *nod* genes [(*nodE,F,G,H*), also known as *hsn*], are implicated in host specificity; although mutations in these loci also affect hair-curling and infection thread formation, blocking or delaying nodulation, these mutations cannot be complemented by homologous genes from other *Rhizobium* species (2,3,10). Such mutants can also curl root hairs on certain heterologous legume hosts, unlike wild type *R. meliloti* (2). In

R. leguminosarum, two additional loci have been identified (*nodI,J*) downstream of *nodDABC* and there is evidence for homologous sequences in *R. meliloti*. The corresponding mutants have not been reported for *R. meliloti* but *R. leguminosarum nodI* and *nodJ* mutants do not have very pronounced deficiencies (18).

We have been studying another class of nodulation-deficient *R. meliloti*, namely, mutants (*exo*) lacking the acidic extracellular polysaccharide (EPS; 8,12). Mutants in *exoB* are deficient in root-hair curling and infection thread formation, but unlike *nod* mutants they do induce nodule formation on alfalfa. Shepherd's crooks occur at extremely low frequency, relative to wild type; some regions along the root show no hair-curling, others show hyper-curling; no infection threads have been seen in *exoB*-induced nodules or in root-hairs of seedlings infected with *exoB* mutants (8; A. M. Hirsch, pers. comm.) Nodules induced by *exo* mutants contain no intracellular bacteria and do not fix nitrogen (Fix$^-$). A few bacteria, which have evidently entered through the epidermis rather than through root hairs, are seen in intercellular spaces.

We investigated the involvement of the *nod* genes in intercellular nodulation by *exo* mutants, by examining the nodulation response of *nod exo* double mutants. We wondered whether genes which were implicated in root hair curling and infection thread development were required for induction of nodules which apparently form independently of these processes. We report here that all the *nod* genes required for root hair entry are indeed also required for intercellular nodulation by *exoB* mutants.

MATERIALS AND METHODS

<u>Genetic Manipulations:</u> (See Table 2.1). Strains were constructed by transduction with φM12, as described (7). Selection was for resistance to neomycin (Nm^r; 100 μg/ml) of Tn*5*, or oxytetracycline (Ot^r; 0.5 μg/ml) of Tn*5-132* linked to *exoB355* (4), as indicated in Table 1. Colonies were screened for *exo* on LB agar containing calcofluor-white (Polysciences, Inc.) at 0.02% and 10mM HEPES buffer, pH 7.4, under long-wave UV light. *Exo*$^+$ colonies fluoresced bright blue-green on this medium; *exo* were non-fluorescent (12).

Plasmids pRmSL26 (13) and pRmC36 (M. Honma, pers. comm.) were mobilized into *R. meliloti* from *E. coli* in a triparental mating using plasmid pRK2013 (6a) or plasmid pRK600 (9).

LacZ-fusion strains were constructed as follows: the 8.7 kb fragment obtained from *Eco*RI digestion of plasmid pRmSL26 (13) was cloned into the chloramphenicol resistance (Cm^r) gene on plasmid pGW1574 (pACYC174 with Nm^r fragment cloned in place of resistance to tetracycline (Tc^r; Steve Winans, pers. comm.) to make plasmid pSR3. This was partially digested with *Sau*3AI and full-size, linear molecules were ligated to the 8.2 kb *Bam*HI fragment from plasmid pGS100 (DeVos, pers. comm.) to produce *lacZ* transciptional fusions, which were tagged by the gene for resistance to gentamicin and spectinomycin (Gm^rSp^r) from pGS100. $Gm^rSp^rNm^r$ transformants were screened by restriction enzyme digestion of plasmid DNA. Plasmids with *lacZ* inserts within the 8.7 kb *R. meliloti* DNA were chosen for further study. These plasmids were mobilizable by pRK600 (9), apparently from the *bom* site of pACYC184, and were thus introduced into *R. meliloti* Rm5000, with selection for Gm^r Sp^r (25 and 100 μg/ml, respectively) and counterselection with rifampicin

(Rf, 50 μg/ml). Since pACYC184-derivatives cannot replicate in *R. meliloti*, all Gm^rSp^r colonies obtained were presumed to have recombined into the *R. meliloti* megaplasmid pRmeSU47a by homology to *nod*. Approximately 1% of Gm^rSp^r colonies were Nm^s, indicating a double homologous crossover and loss of the vector DNA. All of the inserts obtained in this manner were screened for nodulation phenotype. Only one proved to be Nod^-, and the insert was mapped by restriction enzyme analysis to a region including *nodA* and *B* (11). No induction of this fusion by root exudate or luteolin, which have been shown to induce *nodA,B*, and *C* (14,16), could be demonstrated, probably owing to the high background activity associated with the *lacZ* fragment. Other inserts had a Fix^- phenotype, even in the exo^+ background, and are probably inserts in *fixF*, which is located in the same 8.7 kb *Eco*RI fragment as *nodDABC* (1). This work was not pursued further, as successful studies were published by other workers (op cit.). Nonetheless, all of the inserts obtained were tested for nodulation phenotype in *exo* background.

Plant assays: Nodulation tests were performed on *Medicago sativa* cv. Iroquois on nitrogen-free Jensen's agar (20). Seedlings were placed individually in test tubes or up to ten seedlings were tested together on large, square petri dishes (from Vangard Intl. Inc.). Healthy, green, tall plants were scored as Fix^+; Fix^- plants were yellow and stunted.

Table 2.1: Bacterial Strains and Plasmids:

Strain	Relevant Genotype	Source or Reference
R. meliloti		
RCR2011	wild type (≡SU47)	lab collection
Rm1021	SU47 *str-21*	F. Ausubel
Rm5000	SU47 *rif-5*	
Rm5078	Rm1021 *exoB355* Ω5006::Tn*5-132*	(4)
Rm5079	Rm1021 Ω5007 Tn*5-132*	(9)
GMI214	SU47 Rf^r Δ42 (Nod^-) Nm^r	J. Denarie
GMI357	SU47 Rf^r ΔHG0.1 (Nod^-) Nm^r	",(19)
GMI361	SU47 Rf^r ΔHG1.4 (Nod^-) Nm^r	",(19)
Rm6721	Rm1021 Ω5007::Tn*5-132* ΔHG0.1	φM12:GMI357 to Rm5079
Rm6724	Rm1021 *exoB355* Ω5006::Tn*5-132* ΔHG0.1	φM12:GMI357 to Rm5078
Rm6722	Rm1021 Ω5007::Tn*5-132* ΔHG4.1	φM12:GMI361 to Rm5079
Rm6725	Rm1021 *exoB355* Ω5006::Tn*5-132* ΔHG1.4	φM12:GMI361 to Rm5078
Rm6778	GMI214 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to GMI214
Rm6779	GMI214 Ω5006::Tn*5-132*	φM12:Rm5078 to GMI214
RM3600	Rm1021 Δ36 (Nod^-) Nm^r	M. Honma
Rm4400	Rm1021 Δ44 (Nod^-) Nm^r	"
Rm6837	Rm3600 Ω5006::Tn*5-132*	φM12:Rm5078 to Rm3600
Rm6838	Rm3600 Ω5006::Tn*5-132* *exoB355*	"
Rm6839	Rm4400 Ω5006::Tn*5-132*	φM12:Rm5078 to Rm4400
Rm6840	Rm4400 Ω5006::Tn*5-132* *exo*B355	"
S2A3	Rm1021 *fixF*::Tn*5*	S.R. Long (11)
S159	Rm1021 ΩTn*5* (Nod^+)	"
S1B2	Rm1021 ΩTn*5* (Nod^+)	"
S167	Rm1021 ΩTn*5* (Nod^+)	"

Table 2.1 continued

Strain	Relevant Genotype	Source or Reference
S157	Rm1021 ΩTn*5* (Nod$^+$)	"
S1A8	Rm1021 ΩTn*5* (Nod$^+$)	"
S9B2	Rm1021 ΩTn*5* (Nod$^+$)	"
S161	Rm1021 ΩTn*5* (Nod$^+$)	"
S9B8	Rm1021 *nodD*::Tn*5*	"
S1A3	Rm1021 *nodA*::Tn*5*	"
S2B2	Rm1021 *nodB*::Tn*5*	"
S170	Rm1021 *nodC*::Tn*5*	"
S8A2	Rm1021 *nodC*::Tn*5*	"
S9B6	Rm1021 ΩTn*5*	"
Rm6764	S2A3 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S2A3
Rm6765	S159 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S159
Rm6766	S1B2 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S1B2
Rm6767	S167 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S167
Rm6768	S157 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S157
Rm6769	S1A8 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S1A8
Rm6770	S9B2 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S9B2
Rm6771	S161 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S161
Rm6772	S9B8 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S9B8
Rm6773	S1A3 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S1A3
Rm6774	S2B2 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S2B2
Rm6775	S170 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S170
Rm6776	S8A2 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S8A2
Rm6777	S9B6 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to S9B6

Table 2.1 continued

Strain	Relevant Genotype	Source or Reference
GMI5393	RCR2011 *nodE*::Tn*5*	J. Denarie (2)
GMI5117	RCR2011 *nodE*::Tn*5*	"
GMI5140	RCR2011 *nodH*::Tn*5*	"
DEK5	Rm1021 *nodF*::Nm^r	F. Debelle
DEK7	Rm1021 *nodG*::Nm^r	"
Rm6870	GMI5393 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to GMI5393
Rm6871	GMI5393 Ω5006::Tn*5-132*	"
Rm6872	GMI5141 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to GMI5141
Rm6873	GMI5141 Ω5006::Tn*5-132*	"
Rm6876	GMI5394 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to GMI5394
Rm6877	GMI5394 Ω5006::Tn*5-132*	"
Rm6921	DEK5 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to DEK5
Rm6923	DEK7 Ω5006::Tn*5-132* *exoB355*	"
RmD1D2D3	Rm1021 *nodD1*::Tn*5* *nodD2*::Tp^r *nodD3*::Sp^r	M. Honma
Rm6925	RMD1D2D3 Ω5006::Tn*5-132* *exoB355*	φM12:Rm5078 to RmD1D2D3

Table 2.1 continued

Strain	Relevant Genotype	Source or Reference
E. coli		
HB101	*F*$^-$ *hsdS20* (r_B^-,m_B^-) *recA13 ara-14* *proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5* *mtl-1 supE44*	lab collection
MM294A	*pro-82 thi-1 hsdR17 supE44*	lab collection
YMC9	MM294A Δ*lac-pro*	L. Guarente
Plasmids		
pRK2013	Nm^r, Col E1 replicon, RK2 transfer	(6a)
pRK600	Cm^r, Col E1 replicon, RK2 transfer	(9)
pRmC36	Tc^r, *nod-hsn* region	M. Honma
pRmSL26	Tc^r, *nod* region	S. Long, (13)
pSR3	Nm^r, subclone from pRmSL26 Col E1-type replicon	this work

RESULTS

<u>Nodulation response of Δ*nod*,*exo* mutants:</u> We investigated whether the known clusters of *nod* genes on megaplasmid pRmeSU47a were required for nodulation by *exoB* mutants. Strains were constructed which carried both the *exoB355* mutation and one of the following deletions of the *nod* region: Δ36a or Δ44a (Mary Honma, pers. comm.), both extending over the *nod-hsn* region; ΔHG0.1, extending over the common-*nod* genes; ΔHG1.4, extending over the entire area covered by plasmid pRmSL26; or Δ42, a 290 kb deletion in pRmeSU47a (J. Denarie, pers. comm. and ref 19). All of these deletions were Nod$^-$ in the *exo* background, as well as in the *exo*$^+$, indicating that the nodules elicited by *exo* mutants require *nod*$^+$-encoded functions. Occasionally, white, tumorous protrusions were seen on some of the plants inoculated with these deletion mutants in *exo*$^+$ or *exo* backgrounds.

Plasmid pRmSL26 (13) covers *nodDABC*, and plasmid pRmC36 (M. Honma, pers. comm.), covers the entire Δ36 deletion including both *nodDABC* and *nodGFEH*. Both plasmids were introduced into isogenic strains containing the Δ36 or Δ44 deletions and *exoB355* or *exo*$^+$, and plasmid pRmSL26 was introduced into strains carrying the deletions ΔHG0.1, ΔHG1.4 and Δ42. Plasmid pRmSL26 complemented deletions ΔHG0.1 and ΔHG1.4 (but not Δ42) to Nod$^+$, whether in the *exo* or the *exo*$^+$ background, although nodulation by ΔHG1.4(pRmSL26) strains was slightly delayed. Plasmid pRmC36 complemented deletions Δ36 and Δ44 to Nod$^+$, whether in the *exo* or the *exo*$^+$ background.

Thus each of the deletions conferred the same Nod$^-$ phenotype regardless of whether the strain was *exo*$^+$ or *exo*.

<u>Nodulation response of *nod exo* double mutants:</u> We investigated whether individual *nod* genes were required for nodulation by *exoB* mutants, as follows. A series of double *nod exo* mutants was constructed, by introduction of *exoB355* into *nod*-insertion mutants. There was no difference in nodulation response of single *nod* and double *nod exo* mutants on alfalfa (Table 2), either in terms of the number of plants nodulated, or in terms of the number of total nodules (per 10 plants). In some cases (*nodE,F,*and *G*), the *nod exo* strains were slightly more delayed in nodulating as compared to the *nod* single mutant, but this could be ascribed to the delay often seen for single *exo* mutants. A few large, white, nodule-like projections were seen on roots of plants inoculated with RmD1D2D3 (which has inserts in each of the three sequences, *nodD1*, *nodD2* and *nodD3*, M. Honma, pers. comm.), as early as 11 days after inoculation. By seven weeks post-inoculation, numerous small, white or brown nodules were seen on several of the plants inoculated with RmD1D2D3. Only 1 small nodule was seen on the RmD1D2D3*exoB355*-inoculated plants, at seven weeks. In all cases of *exo* genotype, where nodules were seen these were indistinguishable from nodules induced by *nod*$^+$*exo* mutants, i.e. the nodules were white or brown, often very small, and Fix$^-$ (8,12).

Thus, as with the *nod* deletions, individual *nod* mutants had the same phenotype in both *exo*$^+$ and *exo* backgrounds.

<u>Nodulation response of inserts in *nod*-region in *exo* background:</u> A number of Tn*5* inserts in the vicinity of the "common-*nod*" genes which do not themselves convey a Nod$^-$ phenotype (S2A3, S159, S1B2, S167, S157, S1A8, S9B2, S161, S9B6; see Table 2.1 and ref. 11) were also tested in

the *exo* background, in a search for genetic loci which might be required for intercellular nodulation but not for infection-thread-mediated invasion. None of these inserts had any effect on nodulation in the *exo*$^+$ or *exo* backgrounds. Similar results were obtained with transcriptional *lacZ*-Sp^{r}Gm^r fusions in the 8.7 kb *nod*-region. One insert, (Ω125) mapped to *nodA* or *B*, was Nod$^-$ in both *exo*$^+$ and *exo* backgrounds. The other inserts obtained were Nod$^+$ in both backgrounds.

Table 2.2: Nodulation response of *nod exo* mutants:

Strain	Relevant Genotype	Number of plants (out of 10) with nodules at 7 days	11 days	21 days
S1A3	*nodA*	0	0	0
Rm6773	*nodA exoB355*	0	0	0
S2B2	*nodB*	0	0	0
Rm6774	*nodB exoB355*	0	0	0
S8A2	*nodC*	0	0	1[a]
Rm6776	*nodC exoB355*	0	0	0[b]
RmD1D2D3	*nodD1D2D3*	0	2	4[c]
Rm6925	*nodD1D2D3 exoB355*	0	0	0
GMI5092	*nodE*	2	3	8
Rm6870	*nodE exoB355*	0	0	5
DEK5	*nodF*	1	1	7
Rm6921	*nodF exoB355*	0	0	6
DEK7	*nodG*	4	9	9[b]
Rm6923	*nodG exoB355*	0	0	10
GMI5376	*nodH*	0	0	0
Rm6872	*nodH exoB355*	0	0	1
Rm1021	wild type	3	8	10
Rm5078	*exoB355*	3	6	9

[a] Only four plants survived on this plate. The single nodulated plant had one pink nodule, only, which may have been due to a revertant. In other experiments, this locus was completely tight.

[b] Only nine plants survived on these plates.

[c] Nodules were large, white and tumorous-looking.

DISCUSSION

We have shown that all the genes on the nodulation megaplasmid pRmeSU47a required for nodulation by wild type (*exo*$^+$) are also required for nodulation by *exo* mutants of *R. meliloti* SU47. Single inserts in *nodA,B,C,E,F,* and *H* and the triple insert *nodD1D2D3*, prevent or retard nodulation by *exo* mutants to at least the same extent as nodulation by *exo*$^+$ bacteria. We saw no effect of an insert in *nodG*, in either *exo*$^+$ or *exo* background, although Horvath et al. reported a slight delay in nodulation for such mutants (10).

Mutants in *exoB* are affected in hair-curling and infection thread formation (8,12; A. M. Hirsch, pers. comm.). Nodulation of *Neptunia oleracea* by *R. meliloti* does not involve hair curling and infection thread formation and also requires the common *nod* genes *nodA,B,* and *C* (2). However, in that case, the *hsn* genes (*nodE,F,H*) are not required. Similarly, nodulation of *Arachis hypogaea* by *Bradyrhizobium* sp. (*Vigna*) proceeds via intercellular invasion and also requires *nodA*, *B* and *C* (15).

One might expect to find genetic loci in *R. meliloti* which are required specifically for invasion via infection threads and not for intercellular invasion. If so, mutants in such genes would be Nod$^-$ for infection thread-mediated nodulation but Nod$^+$ for nodulation which does not require infection thread formation. Therefore, in our system such mutants would be Nod$^-$ in the *exo*$^+$ background but Nod$^+$ in the *exoB* background. We have been unable to identify any loci with those properties. Therefore, either there are no genes (other than *exo*) required specifically for infection thread initiation, or mutants in such genes do not have a simple Nod$^-$ phenotype and have therefore not

been found, despite the intensive efforts of several groups (e.g. 13,2,10). (We have not tested the putative *R. meliloti* homologues of the *R. leguminosarum nodI* and *nodJ* mutants because these are not yet available.)

Normal infection of alfalfa by wild type *R. meliloti* proceeds via root hair curling and infection thread formation, whereas intercellular nodulation by *exo* mutants apparently involves penetration by bacteria between epidermal cells rather than through root hair cells. Our data imply that these two modes occur via a common pathway. Intercellular or "apoplastic" invasion are frequently described as "crack entry", suggesting a passive process. Our results suggest rather that infection by *R. meliloti exo* mutants is an active, enzyme-dependent process which requires the functions of all of the known *nod* genes.

The physiological function(s) of the system defined by *exo* mutants, Exo, in nodulation by *R. meliloti* has not yet been elucidated. One may speculate that Exo directs the specific invasion of rhizobia via root hairs, such that in its absence bacterial invasion can only occur at other, secondarily susceptible sites, namely intercellular junctions. In any case, Nod function is required for induction of nodule formation as well as for bacterial invasion of nodules, whether via root hair or apoplast. Exo function, however, is required for entry via root hairs only, and therefore is also required, directly or indirectly, for the initiation and development of the infection threads via which the rhizobia enter the developing nodules.

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CHAPTER 3: INTERACTION OF NOD AND EXO FUNCTIONS LATE IN ALFALFA NODULATION

INTRODUCTION

Rhizobium meliloti SU47 secrete an acidic extracellular heteropolysaccharide (EPS; Zevenhuisen and Scholten-Koerselman, 1979; Aman et al., 1981; Tolmasky et al., 1982), and EPS-deficient mutants (*exo*) give nodules on alfalfa that do not fix nitrogen (Fix$^-$; Finan et al., 1985; Leigh et al., 1985). Exo$^+$ function is needed for invasion of root hairs (ibid.). It might also be needed for fixation itself, but the Fix$^-$ phenotype might instead result from an earlier defect in nodule development.

Wild-type *R. meliloti* nodulate by inducing a root hair to curl into a "shepherd's crook"; entering the hair within the curl; eliciting a tubular, ramified infection thread down which they travel across cell borders and deep into the root cortex; and finally, after release from the infection thread within a cortical cell, differentiating into a characteristic large elongate "bacteroid" surrounded by a host-derived "peribacteroid membrane". In contrast, *exo* mutants are deficient in curling the root hair, entering the hair, and eliciting the infection thread; within their Fix$^-$ nodules they are found in intercellular spaces only, having invaded in some way that is still uncharacterized. Clearly, these rhizobia need Exo$^+$ function to invade root hairs. However, *exo* nodules might be Fix$^-$ simply because within them the bacteria are not in the right environment to fix nitrogen, namely,

within cortical cells, differentiated into bacteroids and surrounded by a peribacteroid membrane.

We have investigated this by coinoculating alfalfa with both a *nif*$^{+}$*exo* (*nif* = nitrogen fixation) "indicator" strain and a *nif exo*$^{+}$ "helper". Because coinoculation can overcome the defects of other symbiotic mutants (Rolfe and Gresshoff, 1980; Rolfe et al., 1980; W. Szeto, pers. comm.), we thought the coinoculated *nif exo*$^{+}$ might "help" the *nif*$^{+}$*exo* in *trans* to invade a root hair and reach an inner cortical cell. There, given the *nif* mutation of the *exo*$^{+}$ helper, any nitrogen fixation would have to be due to *nif*$^{+}$*exo* indicator.

Here we show that the ability of the *exo*$^{+}$ helper to help the *exo* indicator to invade inner cortical cells and to differentiate there into a nitrogen-fixing bacteroid is dependent on the *nod* genotype of the helper. The *nod* genes seem to exert their effect there by acting in some way on the Exo^{+} symbiotic function that is missing in *exo* mutants.

We have given a preliminary report of some of this work (Klein et al., 1986).

MATERIALS AND METHODS

Bacterial Strains: See Table 3.1.

Media and Growth Conditions: These have been described (Finan et al., 1985; Leigh et al., 1985). Drugs were supplemented as follows: neomycin (Nm), 100 μg/ml; spectinomycin (Sp), 100 μg/ml; oxytetracycline (Ot), 0.5 μg/ml, all from Sigma Chemical Co. Calcofluor-white (Cellufluor) was obtained from Polysciences, Inc., and added to LB agar to 0.02%. LB-calcofluor plates were buffered with HEPES (10 mM, pH 7.4) from Sigma Chemical Co.

Strain Construction: Transduction with ϕM12 has been described (Finan et al., 1984). Rm6906 through Rm6910 were constructed by selection for Sp^r of Tn*5-233*. Rm6776 was constructed by selection for Ot^r of Tn*5-132* which is linked to *exoB* (De Vos et al., 1986), and screening of colonies for lack of EPS fluorescence with calcofluor-white (Leigh et al., 1985).

Nodulations: Seedlings of alfalfa (*Medicago sativa*) were nodulated in tubes on slants of Jensen's agar (Vincent, 1970; Hirsch et al., 1983). Colonies from agar plates were resuspended in sterile water to a density of approximately 10^7 bacteria/ml and 0.5 or 1 ml of the suspension (depending on the experiment) was added to each tube. For coinoculations, the individual coinoculants were mixed together in equal amounts prior to addition to the seedlings and the same final volume of bacterial suspension was added per tube as for single inoculant controls. Changing the proportions of the coinoculant bacteria did not affect the outcome. Each sample was inoculated onto a minimum of ten plants per experiment and was tested in at least two separate

experiments. Plants were assayed for acetylene reduction at intervals between three and six weeks (Meade et al., 1982).

Recovery of Bacteria from Nodules: Nodules were surface sterilized in 20% Clorox, and washed once in sterile water and then several times in LB supplemented with Mg^{++} and Ca^{++}. Some nodules were squashed whole and plated as below; other nodules were sliced in two with a sterile razor blade. One half of the nodule was fixed for electron microscopy; the other half of the nodule was squashed in a solution of LB (Mg^{++}, Ca^{++}) containing 0.3M glucose. Serial dilutions of the squashed nodule mixture in the same medium were plated on LB agar, supplemented with calcofluor-white and/or drugs as appropriate. In some cases colonies from plates without drugs were replicated onto plates containing drugs for strain confirmation. Colonies on calcofluor-white agar were illuminated with long wavelength ultraviolet and scored as "bright" (*exo*$^{+}$) or "dark" (*exo*). Because nodules were halved before squashing, numbers probably represent underrecovery of bacteria.

Microscopy: Nodules were prepared for light and electron microscopy as described by Hirsch et al.(1983).

Table 3.1: Bacterial strains:

Rm1021 str-21 nod$^+$	nif$^+$	exo$^+$		F. M. Ausubel
Rm5020 str-21 nod$^+$	nif$^+$	exoB-355	Ω5004::Tn5-Nm^{r} [c]	Finan et al., 1985
Rm5078 str-21 nod$^+$	nif$^+$	exoB-355	Ω5006::Tn5-132-Ot^{r} [c]	De Vos et al., 1986
Rm7055 str-21 nod$^+$	nif$^+$	exoF::Tn5-Nm^r		Leigh et al., 1985
Rm1491 str-21 nod$^+$	nifH::Tn5-Nm^r	exo$^+$		F. M. Ausubel
Rm6020 str-21 nod$^+$	nifH::Tn5-233-Sp^r	exo$^+$		Devos et al., 1986
Rm1354 str-21 nod$^+$	nifA::Tn5-Nm^r	exo$^+$		F. M. Ausubel
Rm1027 str-21 nodC::ISRM1	nif$^+$	exo$^+$	Tn5-Nm^{r} [d]	Buikema et al., 1983
Rm1126 str-21 nodC::Tn5-Mu-Nm^r	nif$^+$	exo$^+$		"
Rm5610 str-21 nodA::Tn5-Nm^r	nif$^+$	exo$^+$		φM12(S9B8[b] to 1021)[a] M. Williams
Rm5611 str-21 nodB::Tn5-Nm^r	nif$^+$	exo$^+$		" (S2B2[b] to ")[a] "
Rm5612 str-21 nodC::Tn5-Nm^r	nif$^+$	exo$^+$		" (S170[b] to ")[a] "
Rm5613 str-21 nodC::Tn5-Nm^r	nif$^+$	exo$^+$		" (S8A2[b] to ")[a] "

Table 3.1 continued

Strain					
Rm6906 str-21 nodC::ISRM1	nifH::Tn5-233-Sp^r exo$^+$	Tn5-Nm^r [d]		øM12(6020 to 1126)[a]	this work
Rm6907 str-21 nodA::Tn5-Nm^r	nifH::Tn5-233-Sp^r exo$^+$			" (" to 5610)[a]	"
Rm6908 str-21 nodB::Tn5-Nm^r	nifH::Tn5-233-Sp^r exo$^+$			" (" to 5611)[a]	"
Rm6909 str-21 nodC::Tn5-Nm^r	nifH::Tn5-233-Sp^r exo$^+$			" (" to 5612)[a]	"
Rm6910 str-21 nodC::Tn5-Nm^r	nifH::Tn5-233-Sp^r exo$^+$			" (" to 5613)[a]	"
Rm6776 str-21 nodC::Tn5-Nm^r	nif$^+$	exoB-355:5006::Tn5-132-Ot^r	(pPH1JI)-Sp^r	øM12(5078 to S8A2[b])[a]	"

[a] Indicates øM12 transduction, where first strain is donor and second is recipient.

[b] Jacobs et al., 1985

[c] Insert linked to exoB

[d] Chromosomal insert, linked to pyr

RESULTS

Nitrogen Fixation and Nodule Occupancy:

Single Inoculations: The phenotype of each of the strains used is shown in Table 3.2B, lines 1 through 7. By six weeks after inoculation, there was a clear difference between Fix^+ and Fix^- seedlings in the height and color of their tops: Fix^+ plants were tall and green, whereas Fix^- plants were stunted and yellow. There was some leakiness in the *exo* phenotype: in some experiments, up to 10% of plants inoculated with an *exo* strain (Rm5078, Rm5020 or Rm7055) had one or two Fix^+ nodules (among a large excess of Fix^- nodules) and was therefore scored as Fix^+. At three weeks, wild-type-inoculated plants (Rm1021) averaged 4.5 Fix^+ nodules per plant, and *exoB*-inoculated plants (Rm5078) averaged 7.5 nodules per plant, most or all of which were Fix^-. When nodules from inoculation with *exoB* (Rm5078) were squashed, no colonies were recovered from a majority (approximately 85%) of nodules (Table 3.4, line 3). In general, few colonies (less than 20) were recovered from the remaining nodules, although occasionally a larger number (1000 to 2000) was obtained.

Helping of *exo*. (Table 3.2A, lines 1-3): Helping was tested in pairwise coinoculations of "helper" exo^+nif strains, which could provide Exo^+ function but could not fix nitrogen themselves, with "indicator" *exo* nif^+ strains, which lacked Exo^+ function. This was done for *nodA*, *B* and *C*; *nifA* and *H*; and *exoB* and *F*. Surprisingly, nod^+ and *nod* helpers gave very different results.

The *nod*$^+$*exo*$^+$ helper generally did not give Fix$^+$ nodules with either the *nod*$^+$*exo* (line 1) or the *nod exo* (line 2) indicator (except for occasional Fix$^+$ nodules ascribed to leakiness of the phenotype as above). Both coinoculant genotypes were recovered from a small proportion (5 to 10%) of these Fix$^-$ nodules (Table 3.4, lines 5-6). In a representative experiment [Rm6020 (*nod*$^+$*nif exo*$^+$) with Rm5078 (*nod*$^+$*nif*$^+$*exo*)], a total of 25 nodules from six plants were squashed, and both *nif exo*$^+$ and *nif*$^+$*exo* colonies were recovered from two of them. Many of the nodules were tiny, with morphology typical of *exo*-induced nodules (Finan et al, 1985); among the larger, pinkish nodules, the proportion giving both coinoculant genotypes rose to about 25%.

Ultrastructurally, cells of these Fix$^-$ nodules (Fig. 3) were abnormal in two ways. First, although most cells contained only *nifH*-like bacteroids, several cells contained two morphologically distinct bacteroid forms. One form was elongate like wild type (though perhaps slightly thinner), whereas the other was aberrant. The non-elongate form included a variety of shapes, notably very large spherical bacteroids that were quite extraordinary and novel, and senescent ones with electron-dense cytoplasm and little structural integrity (Fig. 4). Second, occasionally a single bacteroid membrane enclosed bacteroids of both forms (Fig. 3). Multiply-enclosed bacteroids are usually not seen in alfalfa nodules.

In contrast, the *nod* helper did give Fix$^+$ nodules with the corresponding indicator (line 3). In a representative experiment [Rm6910 (*nod nif exo*$^+$) and Rm5078 (*nod*$^+$*nif*$^+$*exo*)] both genotypes were recovered from a majority of nodules tested (16 out of 20), the

remainder yielding no colonies (Table 3.4, line 7). In a few of the host cells, some bacteroids had electron dense bodies (Fig. 5). Electron dense deposits were not seen in bacteroids in other cells, but these deposits are not always found with *nifH* mutants (Hirsch et al., 1983).

<u>Helping of *nod*</u>: (Table 3.2A, lines 4-6): Coinoculation with pairs of strains carrying wild or mutant alleles of *nod* (*A*, *B* or *C*) and *nif* (*A* or *H*) genes in all combinations generally gave Fix^+ nodules, in agreement with Rolfe et al., (1980) and W. Szeto (pers. comm.). For the combination *nod* nif^+ with nod^+*nif* (line 4), all plants were nodulated, and half to two-thirds of them fixed nitrogen (Fix^+), depending upon the experiment. As an example, in coinoculation with Rm1126 (*nod* nif^+exo^+, Nm^r) and Rm6020 (nod^+*nif* exo^+, Sp^r), from Fix^+ nodules roughly three times as many Sp^r (nod^+*nif*) as Nm^r (*nod* nif^+) colonies were recovered, whereas from Fix^- nodules nearly all colonies were Sp^r and few if any were Nm^r. In a similar experiment, where coinoculants were Rm1027 (*nod* nif^+exo^+) and Rm1491 (nod^+*nif* exo^+), TEM of the nodules showed only one form of bacteroid within alfalfa host cells. These bacteroids were elongate and individually enclosed within the peribacteroid membrane (Fig. 1).

Coinoculation with *nod* nif^+exo^+ and nod^+*nif exo* (line 5) gave only Fix^+ plants; large pink nodules gave both types of colonies in a ratio of about 3:1 (data not shown). TEM showed elongate bacteroids, some of which contained the electron dense deposits characteristic of *nifH* mutants (Hirsch et al., 1983) (Fig. 2).

<u>Controls</u>: Coinoculation with *nod nif*$^+$*exo*$^+$ and *nod*$^+$*nif*$^+$*exo* bacteria (line 7) consistently gave only Fix$^+$ nodules. This was found for *nodA*, *B* and *C* with *exoB* and *F* (Rm5610, Rm5611, Rm5612, Rm5613 or Rm1126, with Rm5078; Rm5610 with Rm7055.) Plants were green and healthy and had rates of acetylene reduction equal to or better than those for plants inoculated with wild-type.

Coinoculation with wild-type (Rm1021) and a *nod*, *nif*, or *exo* single mutant always gave Fix$^+$ plants (Table 3.2A, lines 8 through 10). There was no indication of interference by any of the mutants. When nodules from coinoculation of wild type (Rm1021) and *exoB* (Rm5078) were squashed, only wild type (*exo*$^+$, Ot^S) colonies were recovered from most (78%) of the nodules. The remaining nodules gave either no colonies or a mixed population with a large excess of wild type (Table 3.4, line 3).

At least one of the coinoculating strains had to be *nod*$^+$ for the plants to be nodulated (Table 3.2A, line 11). Similarly, at least one of the coinoculating strains had to be *nif*$^+$ for the nodules to fix nitrogen (Table 3.2A, line 12).

<u>Triple Inoculation</u> (Table 3.3): The effect of *nod*$^+$ and *nod* helpers was compared with a single indicator in a triple coinoculation. In this case, *nod*$^+$*nif exo*$^+$ (Rm6020) and *nod nif exo*$^+$ (Rm6910) helpers were inoculated together with a *nod*$^+$*nif*$^+$*exo* indicator (Rm5078). The resulting plants were all Fix$^-$. All of the 40 nodules from eight plants were scored for occupancy, with the following results. Four of the nodules (three of them on a single plant) gave all three coinoculant genotypes. One nodule gave both *nod*$^+$*nif exo*$^+$ and *nod*$^+$*nif*$^+$*exo* and another gave both *nod*$^+$*nif exo*$^+$ and *nod nif exo*$^+$, with an excess of

nod^{+}*nif exo*$^{+}$ in both cases. Twenty nodules gave only *nod*$^{+}$*nif exo*$^{+}$, and the remaining 14 nodules gave no bacteria. Ultrastructurally, some of these Fix^{-} nodules (Fig. 6) were similar to Fix^{-} nodules from ineffective double inoculations, having both elongate and aberrant forms (Fig. 3, 4). (Although we did not see both forms in any of the nodules from which we recovered all three genotypes, we believe this was because most of the triple inoculation nodules were senescent within three weeks.)

Table 3.2: Helping of *exo* bacteria:

	INOCULANT(S) "helper" *nod*	*nif*	*exo*	"indicator" *nod*	*nif*	*exo*	PHENOTYPE [a] Percent of plants Nod+	Fix+
A. Coinoculations:								
Helping of *exo*								
1.	+	-	+	+	+	-	100	8
2.	+	-	+	-	+	-	100	0
3.	-	-	+	+	+	-	100	69
Helping of *nod*								
4.	+	-	+	-	+	+	100	69
5.	+	-	-	-	+	+	92	75
6.	+	-	-	-	+	-	100	0
Controls								
7.	+	+	-	-	+	+	100	92 [b]
8.	+	+	+	-	+	+	100	100
9.	+	+	+	+	-	+	100	92
10.	+	+	+	+	+	-	100	92
11.	-	-	+	-	+	-	0	
12.	-	-	+	+	-	-	100	0
B. Single Inoculations:								
1.	+	+	+				100	92
2.	-	+	+				0	
3.	+	-	+				100	0
4.	-	-	+				0	
5.	+	+	-				100	0
6.	-	+	-				0	
7.	+	-	-				100	0

[a] Data from a representative experiment. Fix+ plants reduced acetylene at least 50% as well as control plants inoculated with wild type (Rm1021) and were tall and green. Fix- plants failed to reduce acetylene or reduced acetylene at less than 20% the levels of plants inoculated with wild type and were yellow and stunted.

[b] 30% of plants reduced acetylene at double the rate for single inoculation with wild type.

Table 3.3: Triple Inoculation:

INOCULANT(S)						PHENOTYPE[a]	
"helpers"			"indicator"			Percent of plants	
nod	*nif*	*exo*	*nod*	*nif*	*exo*	Nod$^+$	Fix$^+$
+	-	+)					
		)	+	+	-	100	0
-	-	+)					
+	-	+	+	+	-	100	0
-	-	+	+	+	-	100	80

[a] Results from a representative experiment. Fix$^+$ plants reduced acetylene at least 50% as well as control plants inoculated with wild type. Fix$^-$ plants failed to reduce acetylene.

Table 3.4: Colony recovery:

INOCULANT(S)						PHENOTYPE	PERCENT OF NODULES GIVING: [a]			
nod	nif	exo	nod	nif	exo	Fix	no bacteria	exo^+	exo^-	exo^+ and exo [b]
Controls										
1. +	+	+				+	21	79	0	0
2. +	+	-				-	86	0	14	0
3. +	+	+	+	+	-	+	4	77.5	0	18.5
Helping of nod										
4. +	-	+	-	+	+	+/-	0	100 [c]	0	0
Helping of exo										
5. +	-	+	+	+	-	-	33.3	58.3	0	8.3 (>10:1)
6. +	-	+	-	+	-	-	10	85	0	5 (>10:1)
7. -	-	+	+	+	-	+	20	0	0	80 (3.3:1)
Triple Inoculation										
8. +	-	+)								
		)	+	+	-	-	35	52.5 [d]	0	12.5 [e]
-	-	+)								

[a] Data are from representative experiments.
[b] The ratio of exo^+ to exo is given in brackets.
[c] Fix^+ nodules had approximately 3:1 nif:nod bacteria.
[d] 50% nif, 2.5% nif + nod nif
[e] 10% all three strains, 2.5% nif + exo, 0 nod nif + exo

DISCUSSION

Our results clearly demonstrate that *exo*$^+$ bacteria can in principle help *exo* mutants both to reach inner cortical nodule cells and to fix nitrogen there. However, whether or not helping actually takes place depends upon the *nod* genotype of the helper bacterium: a *nod* *exo*$^+$ bacterium is an effective helper, allowing *exo* mutants to differentiate into nitrogen-fixing bacteroids (Fix$^+$); a *nod*$^+$*exo*$^+$ bacterium is an ineffective helper, and does not allow coinoculated *exo* mutants to differentiate into the nitrogen-fixing form (Fix$^-$; Table 3.2A). Moreover, the ineffectiveness of the *nod*$^+$ helper supersedes effectiveness of the *nod* helper: a triple inoculation of both the ineffective *nod*$^+$ and the effective *nod* helpers together with the *exo* indicator (Table 3.3) results in the formation of Fix$^-$ nodules.

These results are unexpected particularly considering the fact that in single inoculation a *nod*$^+$*exo*$^+$ bacterium, i.e. wild type, clearly is effective. In other words, *nod*$^+$*exo*$^+$ is effective in *cis* but ineffective to provide Exo function in *trans*. We will propose some possible explanations below.

In the ineffective nodules resulting from coinoculation of *nod*$^+$ helper with *exo* indicator, two morphologically distinct bacteroid forms can be distinguished. One of these forms has differentiated abnormally. The simplest interpretation is that the relatively normal-looking bacteroids are *nod*$^+$*nif* *exo*$^+$ helpers which are unable to fix nitrogen by virtue of being deficient for *nif*, whereas the aberrant bacteroids are *exo* . (We are now exploring bacteroid identification by immunogold labelling.) If this is the case , then the *nod*$^+$*exo*$^+$ helper is enabling *exo* bacteria to penetrate inner cortical cells, albeit at low frequency

(Table 3.4), and also in some manner bringing about the aberrant differentiation of those *exo* bacteria, even though it cannot help for nitrogen fixation. This implies that *nif*$^{+}$ rhizobia can reach inner cortical cells within peribacteroid membranes in a differentiated (if aberrant) state and still be unable to fix nitrogen, i.e. that differentiation and fixation can be uncoupled.

However, an alternative interpretation is that both bacteroid forms are in fact of the same genotype, i.e. *nod*$^{+}$*exo*$^{+}$ helper. If so, this implies that the *nod*$^{+}$*exo*$^{+}$ cannot help the *exo* indicator to penetrate inner cortical cells (or a fortiori to differentiate). However, in this case the *exo* indicator would have to be responsible in some way for the aberrant differetiation of the *nod*$^{+}$*exo*$^{+}$ helper.

By either interpretation, this aberrant differentiation could reflect either an interaction late in nodule development or the indirect consequence of an early event.

Thus the facts to be accounted for are: *nod exo*$^{+}$ helps, but *nod*$^{+}$*exo*$^{+}$ does not help, and supersedes *nod exo*$^{+}$ in a triple coinoculation; this effect of *nod*$^{+}$ is seen in *trans* but not in *cis*; and the ineffective coinoculations give two morphologically distinct forms of bacteroid. We will consider three formal categories of model.

1. **Quantity of Exo.** Compared to the *nod exo*$^{+}$ helper, the *nod*$^{+}$*exo*$^{+}$ helper provides either too little or too much Exo function. Because the *nod*$^{+}$*exo*$^{+}$ helper supersedes the *nod exo*$^{+}$ helper, too little Exo function is ruled out. We cannot rule out too much Exo as the explanation, but this seems to us unlikely.

2. **Quality of Exo.** The molecular species that constitutes Exo function differs depending upon whether it is provided by the *nod* or

the *nod*$^+$ helper. For instance, Nod function might modify Exo. The *cis trans* difference might then be explained if a wild type bacterium is able to use Exo prior to its modification by Nod, but when Exo is provided in *trans* it has already been modified and is therefore ineffective in helping. To explore this model, we have compared exopolysaccharide (EPS) produced in *vitro* from *nod* and *nod*$^+$ bacteria and have found no difference (Appendix). However, we are unable to compare bacterial EPS made in the nodule itself, nor do we know that EPS itself constitutes Exo function rather than a related molecule.

3. **Other factors.** Regardless of the quantity or quality of Exo, a *nod*$^+$*exo*$^+$ helper excludes the *exo* indicator from some (unspecified) critical interaction whereas a *nod exo*$^+$ helper cannot. For instance, a *nod*$^+$*exo*$^+$ bacterium may occupy a site on plant cells more efficiently than an *exo* , whereas a *nod exo*$^+$ does not.

Our results imply an *interaction between Nod*$^+$ *and Exo*$^+$ *function*. The effects of this interaction are seen both early in nodule development, at the time of nodule invasion and later, at the time of bacteroid differentiation. This suggests that both *Nod*$^+$ *and Exo*$^+$ *functions are required not only for root hair invasion early, but also, directly or indirectly, for correct differentation of bacteroids late in nodulation*. In both those processes invading bacteria interact with the plant cell plasmalemma: early, at the root hair surface (and then at the middle lamella as well) the plasmalemma invaginates to accomodate the growing infection thread, and later, as rhizobia are released from the infection thread the plasmalemma surrounds them in the form of the peribacteroid membrane. That similarity may be a clue to what those functions are.

Figure 3.1. Cells of Alfalfa Nodule from Coinoculation of $nod^-nif^+exo^+$ and $nod^+nif^-exo^+$ *R. meliloti*.

Nodules were prepared for transmission electron microscopy (TEM) 3 weeks after inoculation. The plant was Fix^+. Cnly one type of bacteroid (bd) singly enclosed by the peribacteroid membrane (pbm) is present. The bar equals 1μm.

Figure 3.2. **Cells of Alfalfa Nodule from Coinoculation of *nod⁻nif⁺exo⁺* and *nod⁺nif⁻exo⁻ R. meliloti.***

Nodules halves were squashed and prepared for TEM 3 weeks after inoculation. The plant was Fix⁺. The bacteroids (bd) were elongate and singly enclosed by a peribacteroid membrane (pbm). Some bd contain electron dense deposits. The bar equals 1 μm.

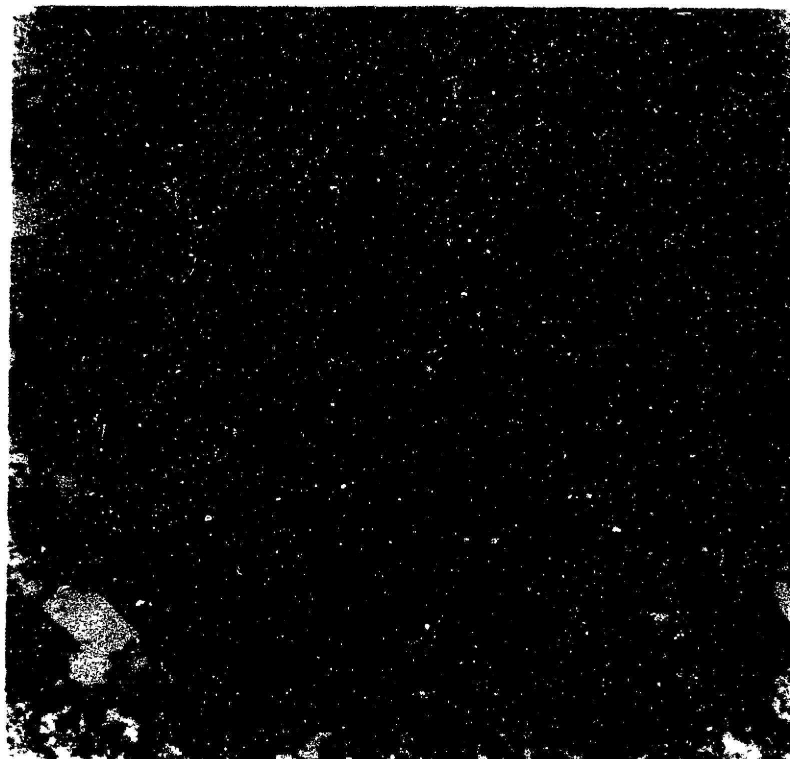

Figure 3.3. Cell of Alfalfa Nodule from Coinoculation of *nod*$^{+}$*nif*$^{+}$*exo*$^{-}$ (Rm5078) and *nod*$^{+}$*nif*$^{-}$*exo*$^{+}$ (Rm6020) *R. meliloti*.

Nodule halves were squashed and prepared for TEM 3 weeks after inoculation. The plant was Fix^{-}. Two types of bacteroids (white and black bd) are present. Occasionally, they are both enclosed within a peribacteroid membrane (pbm). The bar equals 1 μm.

Figure 3.4. Cell of Alfalfa Nodule from Coinoculation of *nod*$^+$*nif*$^+$*exo*$^-$ (Rm5020) and *nod*$^+$*nif*$^-$*exo*$^+$ (Rm1354) *R. meliloti*.

Nodules were prepared for TEM 3 weeks after inoculation. The plant was Fix$^-$. Two types of bacteroids (bd) are present but one (black bd) is elongate and the other (white bd) is senescent. The bar equals 1 μm.

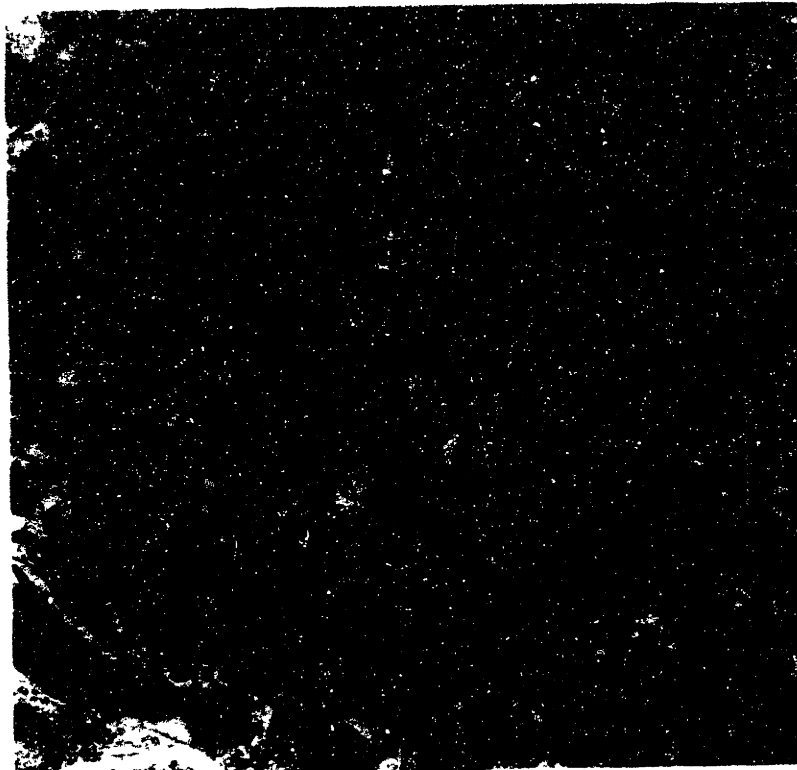

Figure 3.5. Cell of Alfalfa Nodule from Coinoculation of *nod⁻nif⁻exo⁺* and *nod⁺nif⁺exo⁻ R. meliloti*.

Nodule halves were squashed and prepared for TEM 3 weeks after inoculation. The plant was Fix^+. Only one type of bacteroid (bd) is present in nodule cells. A golgi body (g) is in the host cell cytoplasm. The bar equals 1 μm.

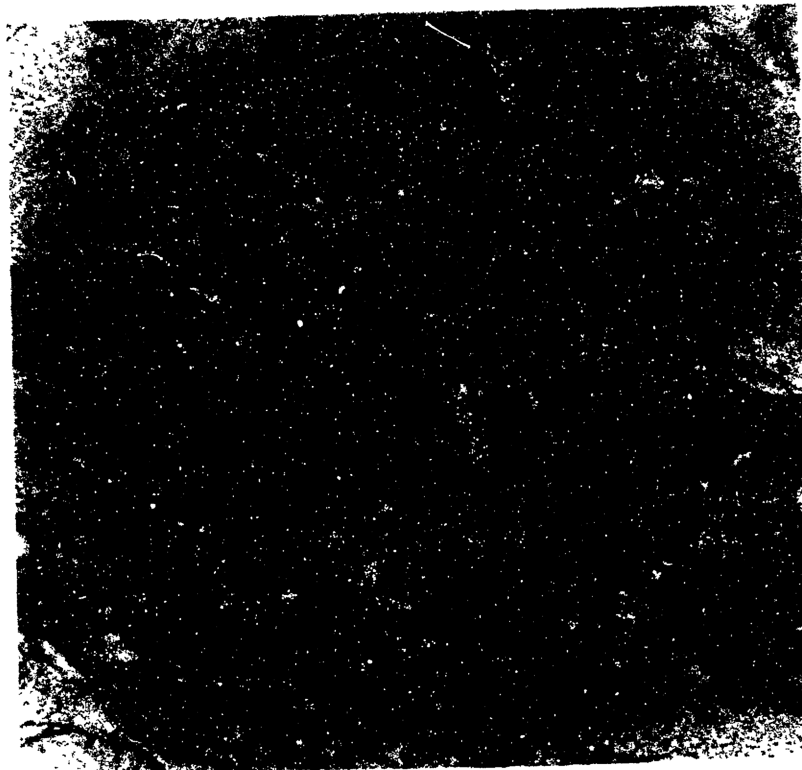

Figure 3.6. Cell of Alfalfa Nodule from Triple Inoculation of *nod*$^{+}$*nif*$^{-}$ *exo*$^{+}$, *nod*$^{-}$*nif*$^{-}$*exo*$^{+}$ and *nod*$^{+}$*nif*$^{+}$*exo*$^{-}$.

Nodule halves were squashed and prepared for TEM twenty days after inoculation. The plant was Fix^{-}. At least two types of bacteroids (white and black bd) are present. The bar equals 1 μm.

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CHAPTER 4: SUPPRESSION OF THE FIX$^-$ PHENOTYPE OF *RHIZOBIUM MELILOTI EXO* MUTANTS

INTRODUCTION

Strains of *Rhizobium meliloti* secrete an acidic extracellular heteropolysaccharide (EPS; Zevenhuisen and Scholten-Koerselman, 1979; Aman et al, 1981; Tolmasky et al., 1982). This EPS binds the fluorescent dye calcofluor-white, and EPS-producing strains fluoresce brightly on calcofluor-containing media.

R. meliloti SU47 is a natural isolate (Vincent, 1941), and EPS-deficient mutants (*exo*), which are dark on calcofluor-containing media, give nodules on alfalfa that do not fix nitrogen (Fix$^-$; Finan et al., 1985; Leigh et al., 1985). *R. meliloti* Rm41 is an independent natural isolate, and its derivative AK631 was isolated originally for its loss of mucoidy (Forrai et al, 1983). We found that AK631 is dark on calcofluor-containing media and is in fact an *exo* mutant, but nevertheless induces Fix$^+$ nodules.

Here, we first establish that the genetic defect which leads to the dark phenotype of AK631 on calcofluor-containing media is indeed in an already-known *exo* locus, *exoB*. We then show that the particular *exo* allele of AK631 (*exoB631*) behaves like other *exo* loci when introduced into the SU47 background, namely, the mutant fails to produce calcofluor-binding material and is Fix$^-$. Conversely, both *exoB* and other *exo* mutations, when introduced into the Rm41 background, are Fix$^+$. Finally, we compare EPS produced by various *exo* mutants in the two backgrounds.

MATERIALS AND METHODS

Media and Growth Conditions: These have been described (Finan et al., 1985; Leigh et al., 1985). Lambda medium (Sabik et al., 1983) was used at full or half strength for growing ϕM12h1; half-strength Lambda medium, supplemented with Mg^{++} and Ca^{++} (2.5 mM each) gave the best growth. Mucoidy was most obvious when colonies were streaked on freshly-poured (within 24 hours) agar. Antibiotics were used at the following concentrations: neomycin (Nm), 100 Mg/ml (for *R. meliloti*) or 50 M/ml (for *E. coli*); kanamycin (Km), 25 Mg/ml (for *E. coli*); spectinomycin (Sp), 100 Mg/ml; oxytetracycline (Ot), 1 Mg/ml ; tetracyline (Tc), 5 Mg/ml; streptomycin (Sm), 100 Mg/ml. Calcofluor-white (cellufluor, from Polysciences, Inc.) was used at 0.02% (Leigh et al., 1985), and LB-calcofluor plates were buffered with Hepes (10 mM, pH 7.4). Colonies on calcofluor were visualized with a hand-held long range UV lamp and scored as *exo*$^+$ ("bright") or *exo* ("dark").

Bacterial Strains and Plasmids: Strains were constructed as indicated in Table 4.1. Transduction and triparental mating have been described (Finan et al.,1984; Finan et al., 1986). Colonies for mating were generally picked directly off a fresh plate and mixed together on LB agar. The patch was then incubated overnight at 30°C, and a toothpickful was streaked onto selective medium.

Rm6856, Rm6857, Rm6860 and Rm6861 were obtained by selection for Ot^r of Tn*5-132* which is linked to *exoB355* (De Vos et al., 1986) and scoring for brightness/darkness on LB-calcofluor. The recipients in these crosses (Rm5209 and Rm5210) are two derivatives of SU47, with Tn*5-oriT* inserted in the same location but in opposite orientations on

pRmeSU47b (:5007::*Tn5-oriT*; Finan et al., 1986). *Tn5-oriT* is a derivative of *Tn5* which contains an origin of transfer (Yakobson and Guiney, 1984). Rm6866 through 6869 were obtained by selection for Nm^r of *Tn5-oriT*, transfer of which required transfer of the entire megaplasmid, and counterselection with Sp^r.

Rm6911 and Rm6912 were obtained by selection for Nm^r of *Tn5* (See Table 4.1). The Nm^r colonies obtained failed to fluoresce on calcofluor-containing medium. Southern hybridization analysis of Rm6912 (Fig. 4.3) confirmed that the *Tn5* insert was in the 8kb fragment of pD56 which contains the *exoF* locus (Sue Long, pers. comm.). Similar analysis verified that the *Tn5* insert in Rm6911 was in the 5.3 kb fragment which contains the *exoA* locus (Fig. 4.4; Sue Long, pers. comm.) Attempts to transduce *exoB*::*Tn5* from Rm7094 into Rm41 were unsuccessful; the few Nm^r colonies that were obtained were all bright on calcofluor-containing medium. Since the Nm^r in Rm7094 is known to be 100% cotransducible with *exoB* (Leigh et al., 1985), these Nm^r colonies probably represented secondary *Tn5* insertions, and were not characterized further.

Plasmids pD56dl and pD56dA were obtained from independent calcofluor-dark colonies which arose at high frequency from the bright diploid, AK631(pD56). The plasmids were mated into *E. coli* MT607 and thence into Rm1021 and SU47, where homologous recombination gave rise to dark colonies, again at high frequency. The dark colonies were purified on LB-calcofluor without Tc, and replicated to check for loss of Tc^r of pLAFR1, which occurred at a frequency of about 5-7%. Rm6903 was obtained in this manner from pD56dl; Rm6904 from pD56dA. In an analogous fashion, the *exoB355* mutation was recombined onto pD56 from

Rm5078. In this case, dark colonies arose at the lower frequency of approximately 0.1%, as had been observed previously (G. De Vos, pers. comm.). These colonies were mated with Rm41, using pRK600 as the mobilizing plasmid, with selection for Tc^r, counterselection by Sp, and screening for dark colonies, which arose at high enough frequency that they could be picked out of a streak of the mating mix. Again, loss of the plasmid occurred (frequency 3-5%) when colonies were purified without Tc pressure, and this procedure gave rise to Rm6919. Similarly, Rm6918 was obtained by mating pD56dl into Rm41, to verify that the multiple phenotype of AK631 (dark on LB-calcofluor; non-mucoid) was due to the mutation at the *exoB* locus. Rm6920 was obtained by mating a derivative of pD56 containing a Tn*5* insert which failed to complement *exoB* mutants (Sue Long, pers. comm.). Although initially all colonies in the first streak from the mating mix were bright, the subsequent streaks (from bright colonies) were predominantly dark. Rm6920 was obtained by loss of Tc^r from one of these dark colonies, was non-mucoid, and was complemented to "bright" by pD56. However, Southern hybridization analysis of Rm6920 DNA, using pD56 as probe, did not show any Tn*5* insert in the region covered by pD56. There is apparently a new allele of *exoB* in Rm6920 (*exoB300*). The differences in plasmid-chromosome recombination frequency for different mutations may reflect local differences in crossing-over, but this has not been pursued further.

Restriction digestion of pD56dl and pD56dA (independent constructs of pD56*exoB631*) showed that two *Eco*R1 fragments (of 1.8 and 4.2 kb) which were present in pD56 were replaced by a single 6 kb fragment; *Cla*I and *Bgl*II digests of pD56*exoB631* were identical to those of pD56.

Southern hybridization (Fig. 4.5) of genomic DNA, using pD56 as probe, showed that this *Eco*R1 polymorphism was present in Rm41 (which is *exo*$^+$) as well as AK631 (=Rm41 *exoB631*) and was therefore not the cause of the *exoB* mutation in AK631. Two independent constructs of Rm1021*exoB631* (Rm6903, Rm6904) had the same (pD56) *Eco*R1 pattern as Rm41, as did a reconstruction of AK631 (Rm6918). During introduction of the *exoB355* allele (see above) into Rm41, the EcoR1 restriction pattern of pD56 from Rm1021 was brought into Rm6919. Thus, there is an *Eco*RI restriction polymorphism linked to the *exoB* locus.

Southern hybridization analysis of Rm6911 (=Rm41 *exoA*::Tn*5*) indicated the presence of another *Eco*RI restriction polymorphism in the region covered by pD34 (*exoA*$^+$; Leigh et al., 1985): a 3.3 kb fragment from *Eco*RI digestion of Rm1021 which hybridized to pD34 was replaced by two smaller fragments, of 1.5 and 1.8 kb, in a digest of Rm41 or Rm6911 DNA, indicating the presence of an additional *Eco*RI site (See Fig.4.4).

<u>Bacteriophage Strains:</u> Most of these have been described (Finan et al., 1984; Finan et al., 1985). ϕM7c is a clear mutant of ϕM7 (T. Finan, pers. comm.). ϕM12h1 is a host-range mutant of ϕM12 (See Appendix).

<u>Plant assays:</u> Seedlings of alfalfa (*Medicago sativa* cv. Iroquois) were nodulated on Jensen's agar (Vincent, 1970; Hirsch et al., 1983) and assayed for acetylene reduction at intervals between three and six weeks (Meade et al., 1982). Each sample was inoculated onto a minimum of ten plants per experiment and was tested in at least two separate experiments.

Bacteriophage sensitivity tests: Phage lysates were spotted onto a lawn of growing bacteria in soft agar. Both bottom and top agar (LB or Lambda) were supplemented with Ca^{++} and Mg^{++} (2.5 mM).

Preparation of EPS and Spectroscopy: EPS was prepared by nitrogen-starvation as described (Leigh et al., 1985), except that cultures were starved for up to 7 days, depending on the experiment. Precipitation with hexadecyltrimethylammonium bromide (cetrimide) was as described (Leigh et al., 1985). Glucose-equivalents were measured by the anthrone method (Loewus, 1962). ^{1}H-NMR-spectroscopy using a 500 MHz spectrometer (at the Francis Bitter National Magnet Lab) was performed at 75°C on total supernatant which had been dialyzed against ultra-pure water, lyophilized and washed in $^{2}H_2O$ three times and resuspended in $^{2}H_2O$. The external chemical-shift standard was sodium 3-(trimethylsilyl)-1-propanesulfonate. The sweep width was 6000 Hz and the relaxation time (RD) was 2 seconds.

DNA Isolation and Southern Hybridization: Plasmid DNA was isolated by the alkaline lysis technique (Maniatis et al., 1982). Chromosomal DNA was isolated by the method of Meade et al. (1982) or the method of Marmur (1961) as modified by G. Barsomian (pers. comm.). Southern transfer and hybridizations were performed according to Maniatis et al. (1982), using GeneScreen or GeneScreenPlus nylon filters.

Table 4.1: Bacterial Strains and Plasmids:

Strain	Relevant Genotype		Source or Reference[a]
R. meliloti			
Rm1021	SU47 *str-21*(Sm^r)	*exo*$^+$	F. Ausubel
SU47	wild type	*exo*$^+$	lab collection
Rm41	wild type, Sp^r	*exo*$^+$	A. Kondorosi
AK631	Rm41	*exoB631*	"
EJ355	SU47 Rif^r	*exoB355*	Finan et al., 1985
Rm5078	Rm1021 :5006Tn*5*-*132*	*exoB355*	De Vos et al., 1986
Rm7031	Rm1021	*exoA*31::Tn*5*	Leigh et al., 1985
Rm7055	Rm1021	*exoF*55::Tn*5*	"
Rm7094	Rm1021	*exoB*::Tn*5*	"
Rm6911	Rm41	*exoA*::Tn*5*	φM12h1:Rm7031 to Rm41
Rm6912	Rm41	*exoF*::Tn*5*	φM12h1:Rm7055 to Rm41
Rm6903,Rm6904	Rm1021	*exoB631*	this work
Rm6913	SU47	*exoB631*	"
Rm6918	Rm41	*exoB631*	"
Rm6919	Rm41	*exoB355*	"
Rm6920	Rm41	*exoB*300	"

Table 4.1 continued

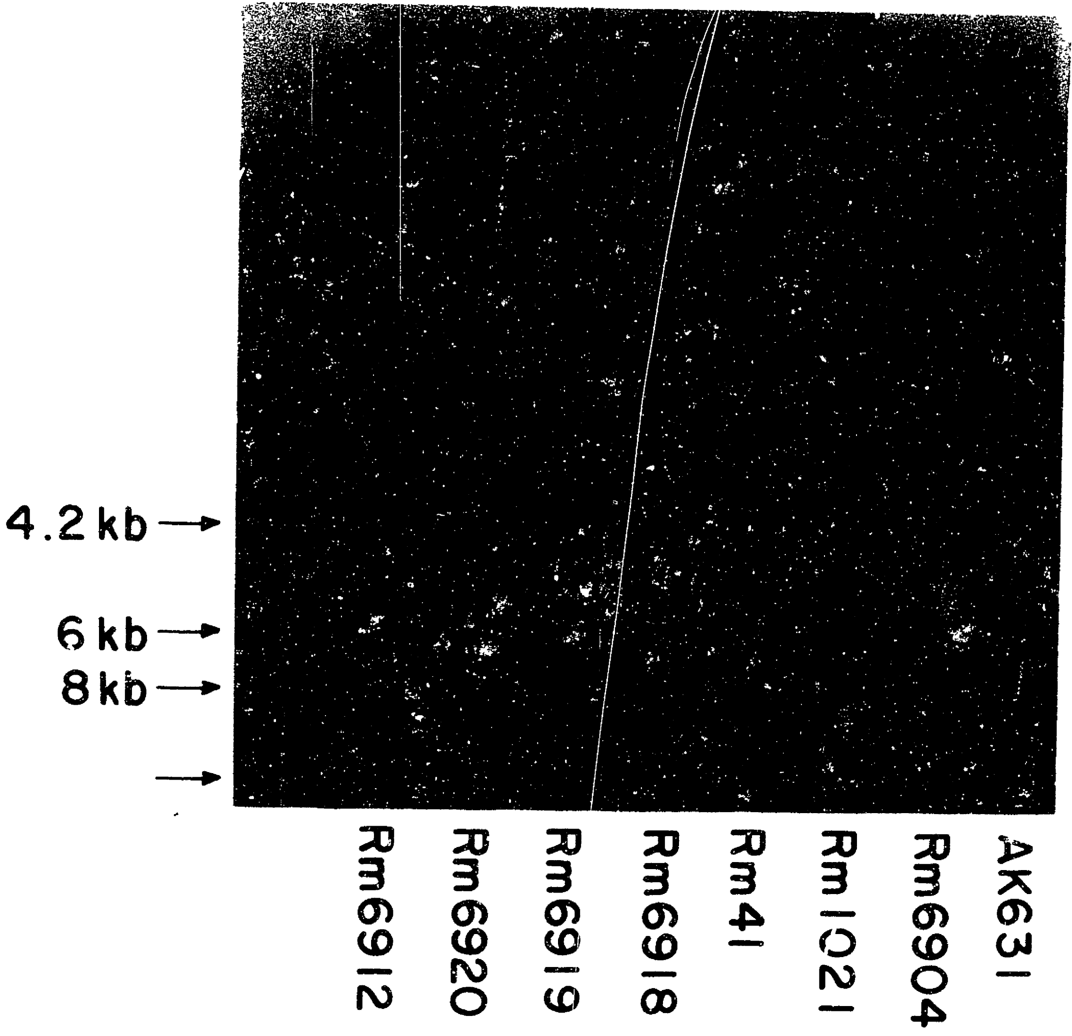
Rm5209	SU47 :5007::*Tn5-oriT*	Finan et al., 1986
Rm5210	SU47 Ω5007::*Tn5-oriT*	"
Rm6856	SU47 Ω5007::*Tn5-oriT* Ω5006::*Tn5-132 exoB355*	φM12:Rm5078 to Rm5210
Rm6857	SU47 Ω5007::*Tn5-oriT* Ω5006::*Tn5-132*	"
Rm6861	SU47 Ω5007::*Tn5-oriT* Ω5006::*Tn5-132*	φM12:Rm5078 to Rm5209
Rm6860	SU47 Ω5007::*Tn5-oriT* :5006::*Tn5-132 exoB355*	"
Rm6866	AK631 pRmeSU47b :5007::*Tn5-ori*	Rm6861 x AK631
Rm6867	AK631 pRmeSU47b :5007::*Tn5-oriexoB355*	Rm6860 x AK631
Rm6868	AK631 pRmeSU47b :5007::*Tn5-ori*	Rm6857 x AK631
Rm6869	AK631 pRmeSU47b :5007::*Tn5-oriexoB355*	Rm6856 x AK631
E. coli		
MM294A	*pro-82 thi-1 hsdR17 supE44*	lab collection
MT607	MM294A *recA56 srl*::Tn*10*	Finan et al., 1986
HB101	*F⁻ hsdS20* (r_B^-,m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44* λ^-	Maniatis et al, 1982
Plasmids		
pD56	*exoB⁺F⁺* Tc^r	Leigh et al., 1985
pD2	*exoB⁺* Tc^r	"
pD34	*exoA⁺* Tc^r	"
pD56 *exoB*314	Tc^r, Nm^r	Sue Long, unpub.
pRK2013	Nm^r, Col E1 replicon with RK2 transfer genes	Figurski and Helinski, 1979

pRK600	pRK2013 Nm^r::Tn*9*, Cm^r	Finan et al., 1986
pD56d1, pD56A	pD56*exoB631*	this work
pD56*exoB355*		this work

[a] φM12 or φM12h1 indicates transduction from first strain (donor) to second (recipient)

x indicates a mating, with pRK2013 or pRK600 as helper.

Fig. 4.3. Southern hybridization showing position of Tn*5* insert in *R. meliloti exoF*::Tn*5* derivative. Genomic DNA was restricted with *Eco*RI and hybridized with ^{32}-P-pD56 DNA. The leftmost lane shows the pattern of Rm6912 (Rm41 *exoF*::Tn*5*); an 8 kb band has been replaced with a 13.7 kb band, indicating that the Tn*5* insert is in the 8 kb fragment. There is an *Eco*RI restriction polymorphism: Rm1021 and Rm6919 (Rm41 *exoB355*) have the SU47 pattern; AK631 (Rm41 *exoB631*), Rm6904 (Rm1021 *exoB631*), Rm41 (wild type), Rm6918 (Rm41 *exoB631*), Rm6920 (Rm41 *exoB300*) and Rm6912 (Rm41 *exoF*::Tn*5*) have a single 6 kb band, in place of the 1.8 and 4.2 kb bands typical of SU47.

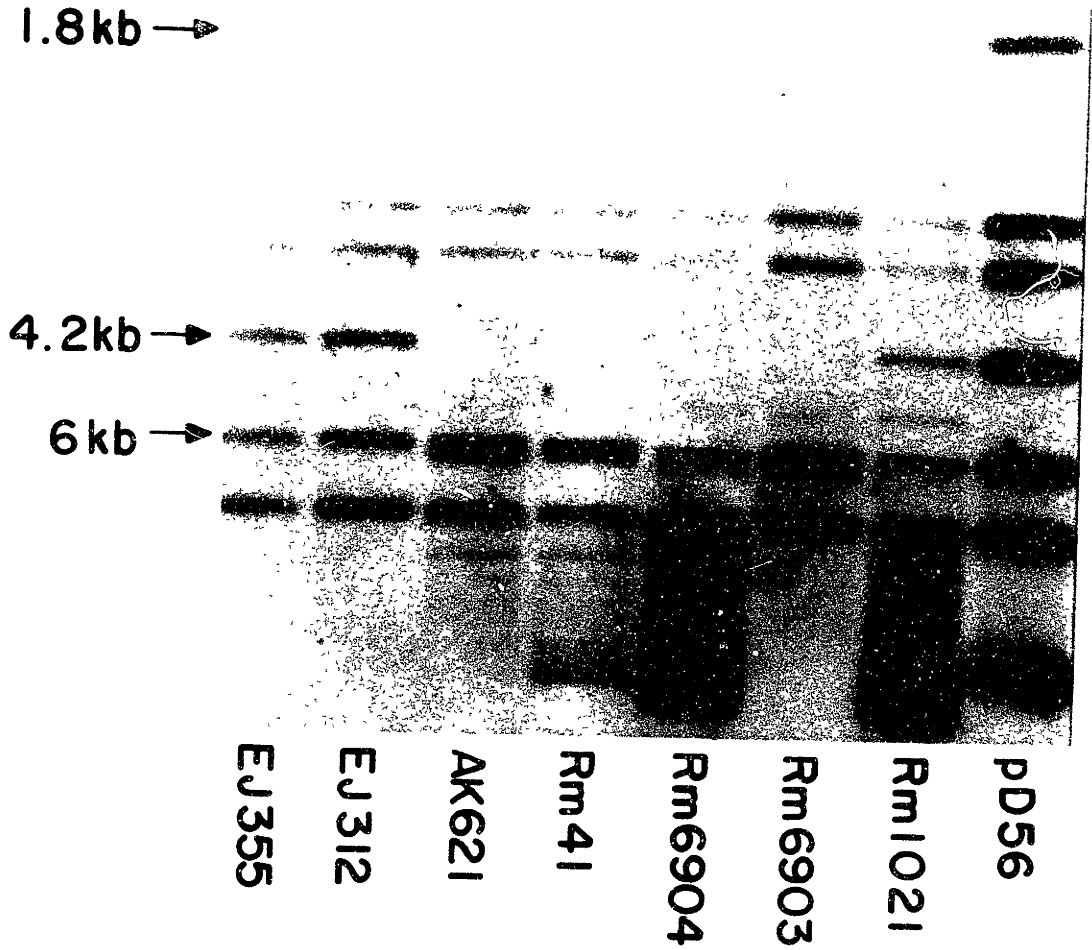


4.2 kb
6 kb
8 kb
Rm6912
Rm6920
Rm6919
Rm6918
Rm41
Rm1021
Rm6904
AK631

Fig. 4.4. Southern hybridization showing position of *Tn5* insert in *R. meliloti exoA*::Tn*5* derivative. Genomic DNA was restricted with *Eco*RI and hybridized with ^{32}-P-pD34 DNA. Both Rm7031 (Rm1021 *exoA*::Tn*5*) and Rm6911 (Rm41 *exoA*::Tn*5*) have inserts in the 5.3 kb fragment. Rm1021 and Rm7031 have a 3.3 kb fragment, which is replaced by two fragments of 1.5 and 1.8 kb in Rm41 and Rm6911, indicating the presence of a restriction polymorphism.

5.3kb

Fig. 4.5. Southern hybridization showing *Eco*RI restriction polymorphism linked to *exoB* locus. Genomic DNA or plasmid pD56 (right lane) was restricted with *Eco*RI and hybridized to ^{32}-P-pD56 DNA. Rm1021 has two bands of 1.8 and 4.2 kb, as does pD56 (which was derived from a library of Rm1021) which are replaced by a single 6 kb band in AK631 (Rm41 *exoB631*), Rm41 and Rm6903 and Rm6904 (independent constructs of Rm1021 *exoB631*).


1.8kb
4.2kb
6kb
EJ355
EJ312
AK621
Rm41
Rm6904
Rm6903
Rm1021
pD56

RESULTS

Complementation of AK631 by *exoB*$^+$-carrying plasmids: *R. meliloti* AK631 has a dark phenotype on calcofluor-containing media, i.e. colonies of AK631 on such media fail to fluoresce brightly under UV light. The parent of AK631, Rm41, is bright (i. e. fluoresces) on such media, as is wild type *R. meliloti* SU47 (Leigh et al., 1985). In order to test whether AK631 was defective in any of the *exo* loci which had been identified in SU47, a series of *exo*-complementing plasmids (ibid.) was mated into the strain. Transconjugants of plasmids pD2 (*exo*B$^+$) and pD56 (*exoB*$^+$*F*$^+$) were bright on LB-calcofluor, whereas transconjugants of other *exo*$^+$-complementing clones [pD5 (*exoD*$^+$), pD15 (*exoC*$^+$) and pD34 (*exoA*$^+$)] were dark. In addition, whereas AK631 accumulated barely detectable levels of cetrimide-precipitable material in cell culture supernatant upon prolonged (about 1 week) nitrogen-starvation, AK631(pD2) accumulated amounts comparable to those of wild type Rm41. Thus, the EPS defect in AK631 falls in the *exoB* complementation group.

Introduction of pRmeSU47b into AK631: In *R. meliloti* SU47, several *exo* loci (including *exoB*) have been mapped to a second symbiotic megaplasmid, pRmeSU47b (Finan et al., 1986). Megaplasmid pRmeSU47b was introduced into *R. meliloti* AK631, as described in Materials and Methods. Two derivatives of SU47 (Rm6857 and Rm6861), with Tn*5*-*oriT* inserted in the same location but in opposite orientations on pRmeSU47b (Ω5007::Tn*5*-*oriT*;Finan et al., 1986), were donors in matings with AK631. In both cases, the majority of transconjugants were bright on LB-calcofluor, indicating that the dark phenotype of AK631 could be corrected by introducing pRmeSU47b*exo*$^+$. When Rm6857 was the donor, less

than 1% of colonies were calcofluor-dark. When Rm6861 was the donor, approximately 15% of colonies were dark; among fifteen of these dark colonies, two were Ot^s. The remaining colonies (dark and bright) were Ot^r, due to a Tn*5-132* insert in the donor, which is linked to the *exoB* locus (See Table 4.1). The dark colonies were presumed to be recombinants of the recipient megaplasmid and megaplasmid pRmeSU47b from the donor. When strains with the *exoB355* mutation on pRmeSU47b (Rm6856=Rm6857 *exoB355*; Rm6860=Rm6861 *exoB355*) were mated with AK631 only dark colonies were obtained. The absence of bright recombinant colonies implies that AK631 behaves like an *exoB* mutant.

<u>Loss of wild type allele in merodiploid:</u> Streaks of AK631(pD56 $exoB^+F^+$) on LB agar containing calcofluor and tetracycline routinely contained a proportion of dark colonies, sometimes as high as 20%. Two such independent dark colonies, as well as a bright control colony, were mated with *E. coli* MT607 and the plasmids obtained were analyzed by restriction endonuclease digestion. The plasmids obtained from dark colonies were named pD56d1 and pD56dA and were presumed to be recombinant for *exoB631*. Both were the same size as the parental plasmid pD56 but were found to have lost a single *Eco*RI restriction polymorphism site (see Materials and Methods). Both these plasmids failed to complement Rm1021*exoB* (Rm7094 and EJ355) but did complement Rm1021*exoF* (Rm7055). The plasmid mated out of a bright colony was indistinguishable from wild type pD56 and complemented both *exoB* and *exoF* strains.

Both pD56d1 and pD56dA were introduced into SU47 and Rm1021 (SU47 *str-21*) where instability was again manifest. Dark colonies could

easily be picked out of a streak of the diploid on LB agar containing both calcofluor and tetracycline.

Three independent bright colonies of AK631(pD56) were grown through five serial cultures in LB broth containing Tc and aliquots were plated on LB calcofluor agar plus Tc after each round of growth. No selective advantage of the dark recombinants was apparent, in liquid; the proportion of dark to bright colonies remained constant (ranging from 0.06 for one colony to 0.25 for another). When bright colonies obtained in this manner were streaked out on the same medium, a proportion of colonies were dark. In contrast, streaks of AK631(pD2= *exoB*$^+$, does not carry *exoF*) were bright throughout.

The instability in this region is probably due to a local hyper-recombinogenic site, possibly similar to one in the *nodC* gene (Jacobs et al, 1985 and S. R. Long, pers. comm.), but this matter was not pursued further.

Bacteriophage Sensitivity: (Table 4.2) *R. meliloti* SU47 derivatives are sensitive to many bacteriophages to which Rm41 derivatives are resistant. Mutants of *exoB* in the SU47 background are resistant to some of the bacteriophages to which the *exo*$^+$ parent is sensitive (namely, ϕM5, ϕM9, ϕM9h1, ϕM10 and ϕM14; Finan et al., 1985; Leigh et al., 1985). AK631 transconjugants of pRmeSU47b (see above), regardless of *exoB* genotype, gained at least some sensitivity to all bacteriophages tested except ϕM12. This implies that sensitivity to the phages tested (with the exception of ϕM12) is at least partially controlled by loci on pRmeSU47b. *ExoB*$^+$ derivatives of Rm41, as well as transconjugants of AK631 containing the *exoB*$^+$-carrying clones pD2 and pD56, were generally more sensitive to ϕM5, ϕM9, ϕM9h1 and ϕM14 than were *exoB* constructs,

which correlates with the phage-resistance seen in *exoB* mutants of *R. meliloti* Rm1021 (op cit.).

<u>Phenotypes of *exo* mutants in different backgrounds:</u> Having established that the calcofluor-dark phenotype of AK631 is caused by a mutation in *exoB* (*exoB631*), we investigated whether the Fix^+ phenotype of this strain was specific to the *exoB631* allele or was a general property of *exo* alleles in the Rm41 background.

<u>*ExoB631* in Rm1021 and SU47 is Fix^-:</u> The *exoB631* allele was introduced (see Materials and Methods) into Rm1021 (Sm^r, non-mucoid) and into its parent, SU47 (Sm^s, somewhat mucoid). In both backgrounds, the mutants (Rm6903, Rm6904 and Rm6913) were indistinguishable from other *exoB* strains: colonies were dark on LB-calcofluor plates; little cetrimide-precipitable material accumulated in culture supernatant even upon prolonged nitrogen starvation (Fig. 4.1); and Fix^- nodules of typical *exo* morphology (i.e. small, round or tumorous-looking, white or brownish; Finan et al., 1985; Leigh et al., 1985) were induced on alfalfa. Rm6913 (SU47*exoB*) formed non-mucoid colonies, although SU47 itself forms mucoid colonies. There was somewhat more leakiness of the Fix^- phenotype in the SU47 than the Rm1021 background: as many as 50% of plants tested were Fix^+, as compared with 15% or less for Rm1021*exo* strains. In all such cases of Fix^+ plants, fixation could be attributed to one or two nodules of wild type appearance, the majority of nodules (usually more than 10 nodules per plant) on the plant having typical *exo* morphology. Other *exo* alleles have not yet been tested in the SU47 (as opposed to Rm1021) background.

ExoB derivatives of Rm41 are Fix$^+$: Two other alleles of *exoB* were tested in the Rm41 background: *exoB355* (Rm6918; Finan et al., 1985; Johansen et al., 1984) and *exoB300* (Rm6920; see Materials and Methods). In addition, *exoB631* was reintroduced into the parental Rm41 strain (Rm6919). The resulting strains, like AK631, were non-mucoid and dark on LB-calcofluor, produced little cetrimide-precipitable material upon nitrogen-starvation (Fig.4.1), and induced the formation of healthy, pink, Fix$^+$ nodules on plants. Evidently, *exoB* mutations in general are Fix$^+$ in the Rm41 background.

ExoA and *exoF* derivatives of Rm41 are Fix$^+$: Rm41*exoA*::Tn*5* (Rm6911) and *exoF*::Tn*5* (Rm6912) colonies on LB-calcofluor agar were dark. Colonies were very mucoid, as for Rm41 itself and unlike Rm41*exoB* derivatives, which are non-mucoid. Little cetrimide-precipitable material was accumulated in culture supernatant upon nitrogen starvation (Fig.4.1). Both Rm41*exoA* and *exoF* induced the formation of healthy, pink, Fix$^+$ nodules on alfalfa. Thus *exoA* and *exoF* mutations too are suppressed for Fix$^-$ phenotype in Rm41.

^{1}H-NMR spectroscopy of supernatant from N-starved cells: To examine EPS, ^{1}H-NMR spectra of dialyzed supernatants from nitrogen-starved cultures (see Materials and Methods) of the following strains were compared (Fig.4.2): Rm41 (wild type), Rm41*exoA*::Tn*5* (Rm6911), Rm41*exoF*::Tn*5* (Rm6912 ; data not shown), and Rm41*exoB355* (Rm6918). No attempt was made to separate large polymers from smaller oligomers. The primary substance in Rm41 supernatant had a similar pattern to that produced by Rm1021 (Leigh et al., 1985; Aman et al., 1981), indicating that the major substance in Rm41 supernatant is the calcofluor-binding EPS which is the product of the *exo* system. However, in supernatants of

the *exoA*::Tn*5* and *exoF*::Tn*5* derivatives the doublet at about 2.5 ppm, due to the methylene protons of the succinyl group of the calcofluor-binding EPS, was missing. In addition, the singlets at about 2.1 ppm and 1.5 ppm, representing acetylation and pyruvylation, respectively, were reduced, relative to the same peaks from Rm41 supernatant. This suggests that an additional substituted EPS is made by Rm41, masked by the predominant calcofluor-binding EPS in wild type Rm41, but detected in Rm41*exoA* or *exoF* mutants which are deficient in production of the calcofluor-binding EPS. All three of the peaks representing acidic substituents were highly reduced or absent in supernatant of Rm6918 (Rm41 *exoB355*). Apparently, production of the non-calcofluor-binding EPS detected in Rm41*exoA* and *exoF* mutants requires ExoB function. The spectrum of Rm6911 (Rm41 *exoA*::Tn*5*) supernatant contrasts with the spectra of supernatants from *exo* mutants in the Rm1021 background: neither Rm7031 (Rm1021 *exoA*::*Tn5*) or Rm6903 (Rm1021 *exoB355*) showed any upfield peaks (data not shown). The carbohydrate material in dialyzed supernatant from nitrogen-starved Rm1021*exo* cultures probably represented non-substituted carbohydrate oligomers (J. Reed, pers. comm.)

Table 4.2: Bacteriophage Sensitivity:

Strain	Relevant Genotype	Response to phage ϕM								
		1	5	7	7c	9	9h1	12	14	12h1
Rm1021	SU47 *str-21*	+++	+++	+	+++	+++	+++	+++	+++	+++
Rm41	Rm41 *wt*	-	+	ND	ND	+	+	±	-	++
AK631*	Rm41 *exoB*	-	-	-	±	±	±	+	-	++
Rm6866	AK631(pRmeSU47b)[1]	+	+	+	++	+++	+++	+	+++	ND
Rm6868	AK631(pRmeSU47b)[1]	+++	+++	+	+++	+++	+++	+++	+++	ND
Rm6867	AK631(pRmeSU47b*exoB*)[1]	+	±	+	++	+	+	+	+	ND
Rm6869	AK631(pRmeSU47*exoB*)[1]	+	±	+	++	±	±	+	+	ND
AK631(pD2)	AK631/B^+F^+	-	±	-	-	+	++	-	$+^P$	ND
AK631(pD56)	AK631/B^+F^+	-	+	-	-	++	++	±	$+^P$	ND**
AK631(pD34)	AK631/A^+	-	-	±	+	+	±	+	-	ND
Rm6911	Rm41*exoA*	-	+	ND	ND	+	±	±	-	++
Rm6912	Rm41*exoF*	-	+	ND	ND	+	ND	ND	-	++

+++ clear spot
+ turbid spot
± very turbid spot
$+^P$ some plaques within spot
\- no lysis
ND not determined

* Rm6918, Rm6919, Rm6920 had same pattern.

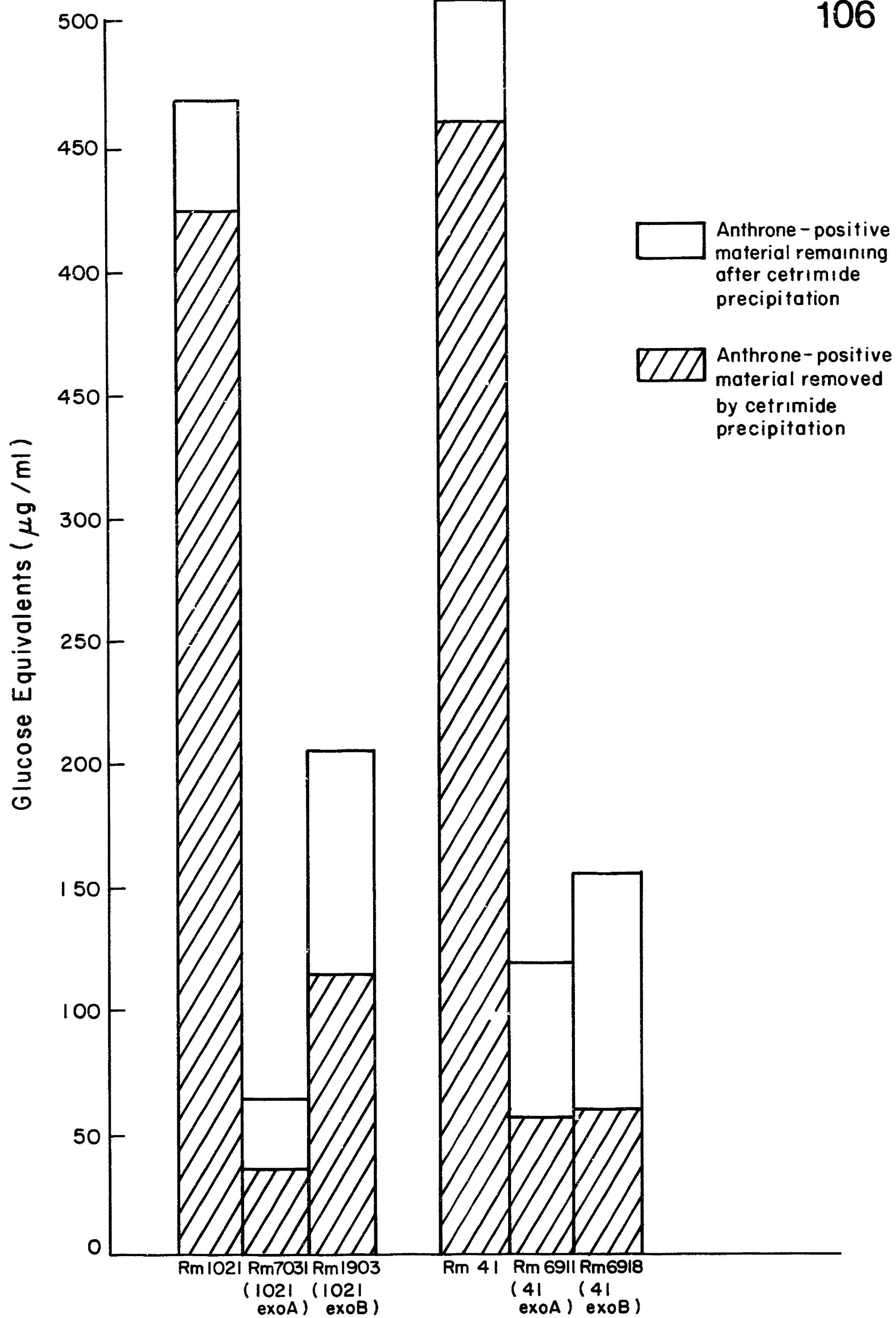
**Rm6920(pD56) gave clear spot.

[1] Rm6866 through Rm6869 are derivatives of AK631 obtained by conjugation of pRmeSU47b from SU47 *exo*$^+$ or *exoB* derivatives with Tn*5*-*oriT* in opposite orientations.

Table 4.3: Phenotypes of *exo* mutants in different backgrounds

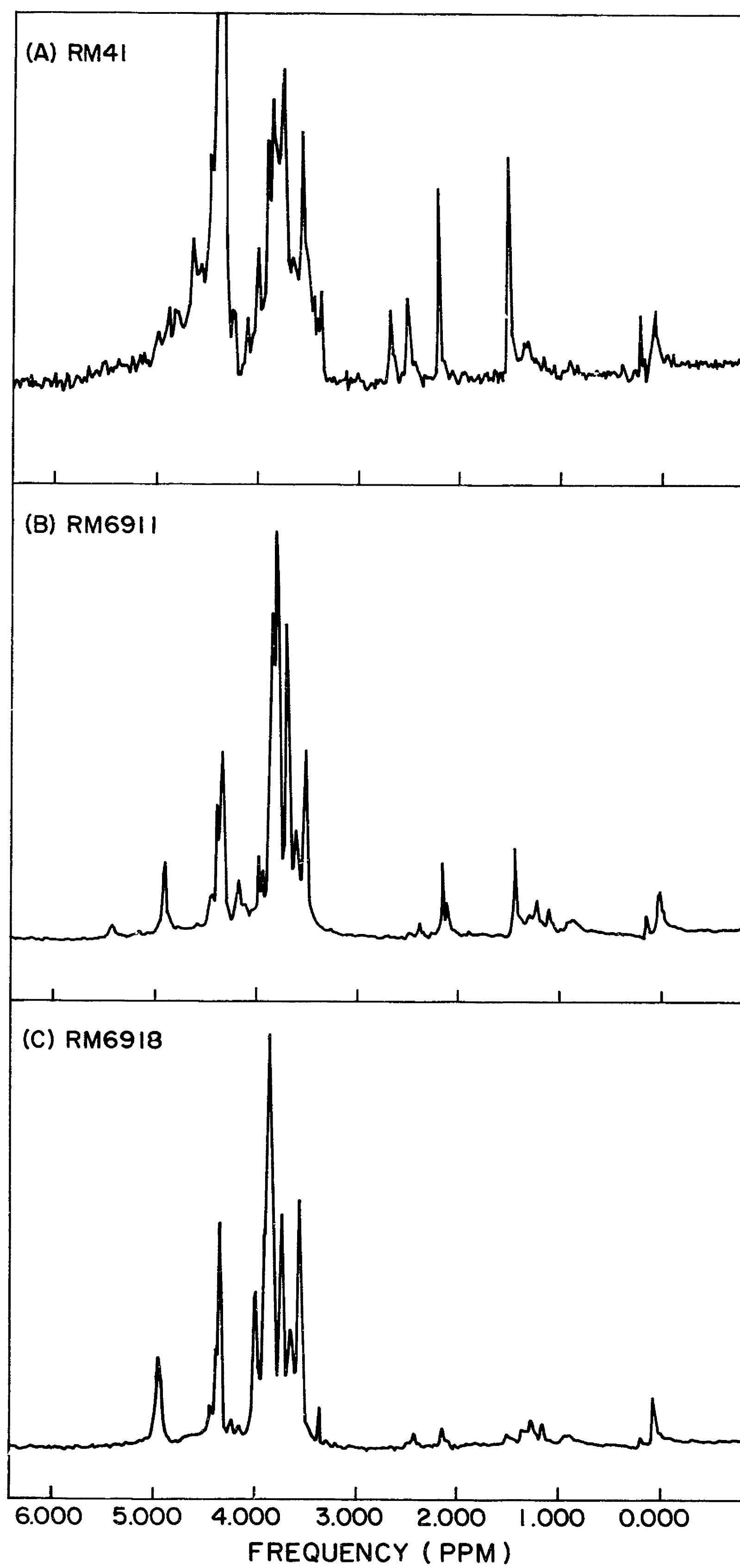
Background	*exo* genotype	Fluorescence on LB-calcofluor	Mucoidy	N_2-Fixation
Rm1021	wt	+	-	+
"	*exoB*	-	-	-
"	*exoA, F*	-	-	-
SU47	wt	+	+	+
"	*exoB*	-	-	-
Rm41	wt	+	++	+
"	*exoB*	-	-	+
"	*exoA, F*	-	+	+

Fig. 4.1. Anthrone tests on *exo* mutants in different backgrounds. Cultures were starved in nitrogen-free medium for two days. Aliquots of culture supernatant were assayed for anthrone-positive material before or after treatment with cetrimide.



Glucose Equivalents (μg / ml)
500
450
400
450
300
250
200
150
100
50
0
Rm1021
Rm7031 (1021 exoA)
Rm1903 (1021 exoB)
Rm 41
Rm 6911 (41 exoA)
Rm6918 (41 exoB)
Anthrone-positive material remaining after cetrimide precipitation
Anthrone-positive material removed by cetrimide precipitation

Fig. 4.2. ^{1}H-NMR spectra of dialyzed supernatants from Rm41 wild type and *exo* mutants. Top panel: Rm41 (wild type). The regions from 3.3 and 4.0 ppm and 4.5 to 4.9 ppm represent the ring protons and β-anomeric protons, respectively, of the sugar constituents. The doublet at 2.5 ppm represents the methylene protons of the succinyl side group. The singlets at 2.1 and 1.5 ppm represent acetyl and pyruvyl groups, respectively. Middle panel: Rm6911 (Rm41 *exoA*::Tn*5*). The doublet at 2.5 ppm (succinyl) is missing and those at 2.1 and 1.5 ppm (acetyl and pyruvyl substituents) are reduced relative to wild type. Bottom panel: Rm6918 (Rm41 *exoB355*). All upfield peaks are highly reduced.



(A) RM41
(B) RM6911
(C) RM6918
6.000
5.000
4.000
3.000
2.000
1.000
0.000
FREQUENCY (PPM)

DISCUSSION

We have shown that several classes of *exo* mutants (*exoA*, *exoB* and *exoF*), when in the *R. meliloti* Rm41 background, induce the formation of Fix^+ (effective for nitrogen fixation) nodules on alfalfa . The same mutants in the SU47 background induce the formation of Fix^- nodules on alfalfa (Finan et al., 1985; Leigh et al., 1985). As in the SU47 background, Rm41*exo* mutants do not produce the calcofluor-binding EPS which was shown in SU47 to be required for effective (i.e. nitrogen-fixing) nodulation. Mutant colonies fail to fluoresce brightly on calcofluor-containing medium, and nitrogen-starved cultures produce little cetrimide-precipitable material. Thus, Rm41*exoA*, *exoB* and *exoF* mutants are suppressed for Fix^- phenotype.

Formally, there are two classes of explanation which could be invoked to explain the Fix^+ character of Rm41 *exo* mutants. Firstly, there might be an alternative mode of nodulation which is independent of EPS. We have no evidence addressing this model. Secondly, there might be an alternative polysaccharide that serves the same function in nodulation as the calcofluor-binding EPS. Production of a second EPS is activated by an additional mutation and is believed to suppress the Fix^- phenotype of *exoA* and *exoF*, but not *exoB*, mutants in the Rm1021 (SU47 *str-21*) background (J. Glazebrook, pers. comm.).

We investigated whether *R. meliloti* Rm41 produced another EPS in addition to the calcofluor-binding EPS. Although we found preliminary evidence for the production of such a non-calcofluor-binding EPS in *exoA* and *exoF* mutants of Rm41, it was not produced by *exoB* mutants of Rm41. Since Rm41 *exoB* mutants are also Fix^+, another suppressor must be present in the Rm41 background.

^{1}H-NMR spectra of dialyzed supernatants from nitrogen-starved cultures of Rm41 *exoA* and *exoF* mutants exhibited singlets with the chemical shifts expected for the acidic substituents, acetyl and pyruvyl, on carbohydrate material. The spectra differed from those obtained from *exo*$^{+}$ cultures in the following respects: there was no evidence of succinylation in the material in the supernatants derived from *exoA* and *exoF* mutants, and the amounts of acetate and pyruvate appeared to be lower than for wild type. There was some indication of differences between wild type and mutant supernatants in the regions of the spectra corresponding to the ring protons (3.3 to 4.0 ppm) and β-anomeric protons (4.5 to 4.9 ppm) of the sugar constituents. Confirmation of such differences will require more detailed structural studies. It is possible that this additional polysaccharide is responsible for the mucoidy of Rm41 colonies. The spectra obtained by ^{1}H-NMR analysis of the second SU47 EPS and dialyzed supernatant from nitrogen-starved cultures of Rm41*exoA* are clearly different (data not shown).

^{1}H-NMR spectra of dialyzed supernatant from nitrogen-starved cultures of Rm41*exoB* showed no peaks corresponding to acetyl, pyruvyl or succinyl substituents. We do not know whether the carbohydrate material in such supernatants corresponds to the oligomeric carbohydrate molecules found in supernatants of Rm1021*exo* mutants (J. Reed, pers. comm.) or whether it represents high molecular weight, unsubstituted polymers. No upfield peaks were seen in dialyzed supernatants from Rm1021 *exoB* or *exoA* mutants (data not shown).

If there is an additional polysaccharide produced by Rm41 derivatives, which we have failed to detect, the regulation of this

putative polysaccharide would be different from that of the calcofluor-binding EPS, such that it is not secreted in large quantities by nitrogen-starved cells. It is possible that EPS production can be induced by the plant. However, we found no evidence for induction of EPS by addition of root exudates to cultures of Rm41*exoB* mutants.

We have constructed a gene library from Rm41 (see Appendix) and are attempting to select plasmids from that library which suppress the Fix$^-$ phenotype of SU47*exo* mutants. Success will require that the necessary genes be encoded within a contiguous 20-25 kb region of DNA. Alternately, larger R-prime derivatives could be mobilized into SU47*exo* mutants. Finally, we could mutagenize Rm41*exo* bacteria and screen the mutants for those which induce nodules of *exo* morphology on alfalfa. If we can establish the mechanism of suppression of the Fix$^-$ phenotype of *exo* mutants in the Rm41 background, we should gain new insight into the molecular processes required for establishment of an effective symbiosis.

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CHAPTER 5: CONCLUDING REMARKS

Nodulation of legumes by rhizobia presents many profound questions to the biologist. How do bacterium and plant recognize one another? What is the basis for the specificity of that recognition? What are the molecular mechanisms which lead to the complex developmental pathway that occurs in nodulation? What are the signals whereby plant and bacteria communicate and how do those signals relate to the temporal and spatial developmental sequence? What can we learn about general plant development from studying nodulation? What can we learn about other bacterial-plant interactions?

In recent years, significant advances have been made in identifying the bacterial genes required for nodule induction and development. Only a few genes appear to be essential for nodule induction (*nodDABC*). Other loci have been identified which affect nodule development,including the *exo* system which is required for the production of an acidic extracellular polysaccharide. In terms of understanding the precise molecular function of the products of the various genes, biologists are just beginning to scratch at the surface of a difficult problem. If we can picture nodule development as a complex jigsaw puzzle, with spatial and temporal dimensions, each of these gene products must fit into that puzzle.

In this study, we find evidence for interlocking of some of the pieces. A schematic diagram (after Debelle et al., J. Bacteriol. 168, 1075-1086) is presented (Fig.5.1). Although we have not yet defined the nature of the interaction, we have shown that Nod function (as defined by *nod* genes) and Exo function (as defined by *exo* genes) do interact and

that interaction affects whether or not Exo function can be provided by one bacterium to another. Further, we show that Nod affects not only the initial stages in nodule development but also the later stages of bacteroid differentiation. This effect could be direct or could result from earlier commitment to a particular developmental pathway. Similarly, we show that Exo function is required not only for invasion via root hairs but also, directly or indirectly, for bacteroid differentiation.

In addition, we have shown that Nod function is required for bacterial invasion of the plant root not only via root hairs but also via apoplast. Exo function, on the other hand, is required for invasion via root hairs only. Exo appears to function in infection thread initiation and/or development.

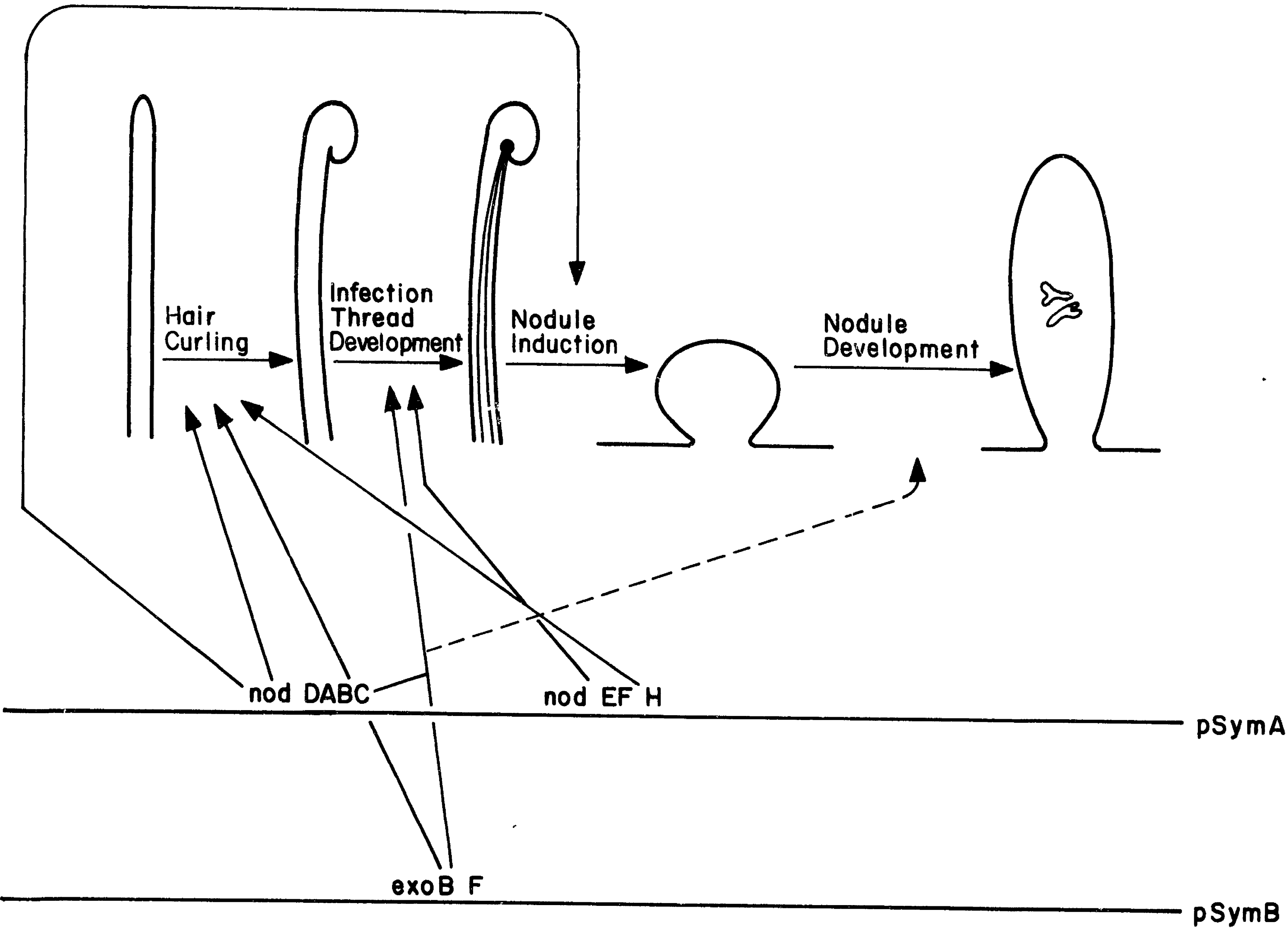
To return to the puzzle analogy, it is clear from this study that not all of the pieces are determinate. Evidently, Exo function can either be bypassed or replaced, since *exo* mutants of *R. meliloti* Rm41 induce normal nodule development. One implication of this finding is that an apparent lack of symbiotic deficiency in polysaccharide-deficient mutants does not necessarily rule out involvement of that polysaccharide in nodulation.

This thesis has raised as many questions about nodule development as it has answered. What is the nature of the Nod-Exo interaction? What is the nature of the natural "suppressor" of the Fix$^-$ phenotype of Rm41 *exo* mutants? What Exo does, what its role is in nodule development, is still unknown. It is not known whether EPS or a related molecule(s) (or both?) constitutes the active principle of Exo function. There is no evidence that EPS has host-specificity, however some

evidence is emerging that *nodD* has a measure of species-specificity (M. Honma, pers. comm.) in its induction, and it is possible that some specificity could therefore be conveyed to EPS via the Nod-Exo interaction. Exo function might be required for discriminating between root hairs and the rest of the epidermis. Exo might be required for root-hair penetration. A buildup of Exo within a curl, or between two juxtaposed root hairs might serve to force bacteria across lesions in the root-hair wall, which may in turn be caused by enzymes of bacterial origin. Exo might regulate the uneven growth that leads to root-hair curling. Exo could form part of the matrix of the infection thread. If EPS has a structural role in the nodule, antibodies to EPS (once these are available) will help to localize EPS in *situ*.

It is clear from this study that the role of Exo in nodulation is complex and requires interaction with, at least, the "common *nod*" genes. It is noteworthy that mutants of *R. meliloti* SU47 which lack the calcofluor-binding EPS are apparently unaffected for non-symbiotic growth, at least in the laboratory. It is possible that the mutants secrete small amounts of other exopolysaccharides. Whether these mutants are less "fit" in their natural habitat has not been investigated.

Fig. 5.1. Stages at which Exo acts in nodulation.


Hair
Curling
Infection
Thread
Development
Nodule
Induction
Nodule
Development
nod DABC
nod EF H
exoB F
pSymA
pSymB

APPENDIX 1: COMPARISON OF EPS FROM *NOD*$^+$ AND *NOD*$^-$ BACTERIA

INTRODUCTION

In Chapter 3, we provided genetic evidence for an interaction of Nod and Exo functions: *nod*$^+$*exo*$^+$*nif* bacteria cannot "help" *nod*$^+$*exo nif*$^+$ indicator bacteria to form effective nodules, whereas *nod exo*$^+$*nif* are competent helpers. Here, we compare exopolysaccharide (EPS) obtained from wild type (*nod*$^+$) and *nod* cells. In order to study EPS produced when *nodABC* are induced, luteolin, which has been shown to induce those genes (Peters et al., 1986), was added to the culture medium. Under the conditions used, we found no difference between EPS obtained from the two sources.

MATERIALS AND METHODS

Bacterial Strains: Rm1021 (SU47 *str-21*), Rm5613 (Rm1021*nodC*::Tn*5*), Rm5078 (Rm1021 *exoB-355* :5006Tn*5-132*), Rm1021(pRmM57=*nodC-lacZ*, Tc^r; Mulligan and Long, 1985).

Production of EPS: EPS overproduction by wild type and *nod* cultures was induced by nitrogen starvation as described (Leigh et al., 1985), with the following modifications: cells were pregrown in M9 medium (Maniatis et al., 1982) and starved in Fahraeus medium (Fahraeus, 1957) supplemented with mannitol (20mM). Luteolin (0.5 μM; from ICN Biomedicals, Plainview NY) was added to growing cells, where applicable, two to three hours before cultures were harvested, washed and resuspended in starvation medium containing luteolin. A culture of Rm1021(pRmM57*nodC-lacZ*) was grown in parallel, and tested for β-galactosidase activity as described (Mulligan and Long, 1985) to ensure that *nod* genes were induced under the conditions used. After two days nitrogen-starvation, cell supernatant was collected, dialyzed against ultra-pure water, and lyophilized and washed in ^2H_2O two or three times. ^{1}H-NMR spectroscopy was performed on the sample, using a 500 MHz NMR spectrometer. The same samples were then sterilized with 50% ethanol, followed by evaporation and resuspension in sterile water.

Plant assays: Sterile seedlings of *Medicago sativa* var. Iroquois on slants of Jensen's agar (Vincent, 1970) were inoculated with 0.5 ml of a suspension of bacteria in sterile water ($\approx 10^7$/ml). 20 μl of an EPS suspension ($\approx$2 mg/ml) were added and the tube gently tilted so as to coat the root. Seedlings were incubated at 20C, with 16 hours of light

in every 24 hour cycle. Twenty seedlings were tested, for each treatment. Plants were judged to be Fix^+ when seedlings were green and tall; they were judged to be Fix^- when yellow and stunted.

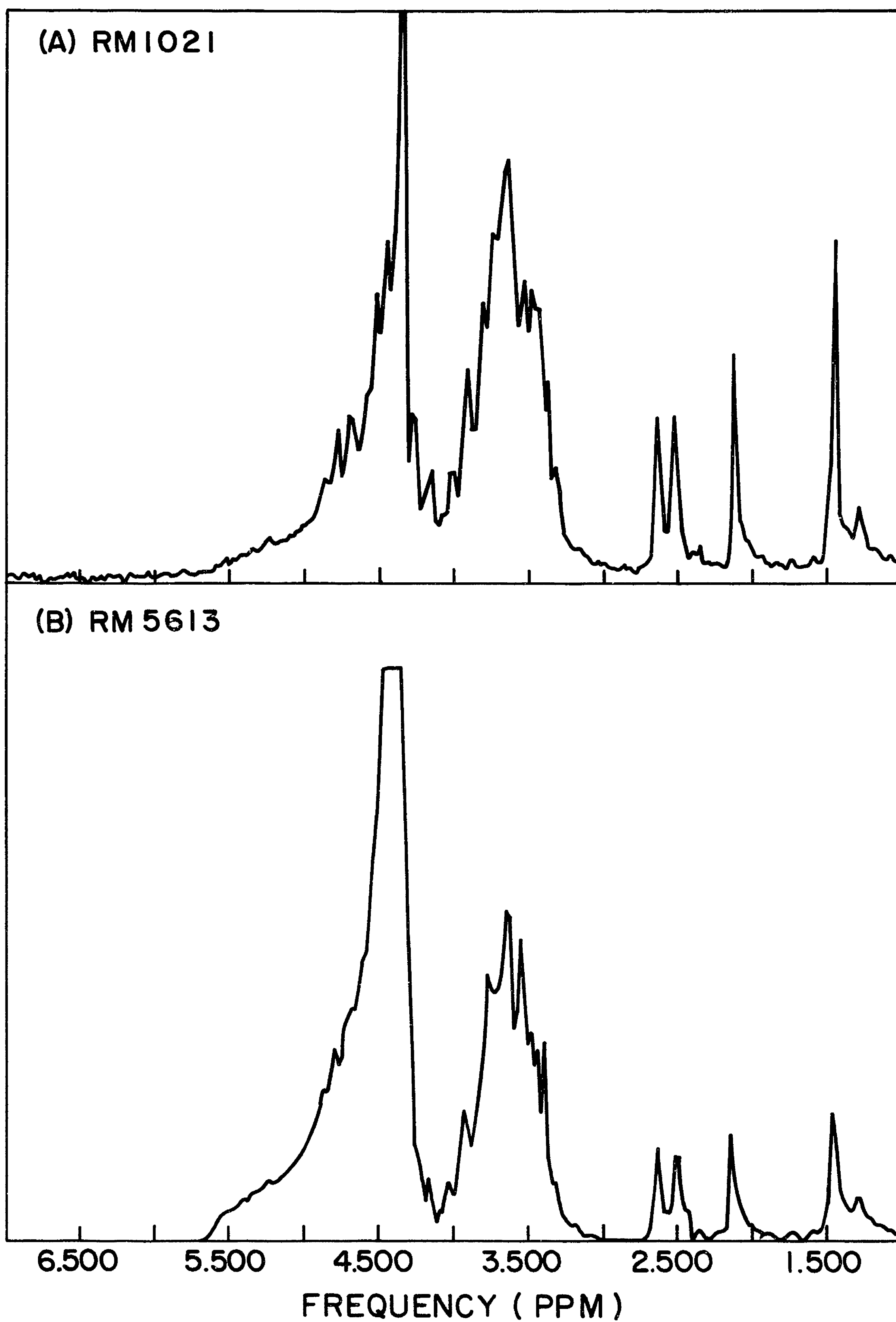
RESULTS

Comparison of ^{1}H-NMR spectra of EPS from wild type and *nod* cells: There was no discernible difference between the spectra of dialyzed supernatants obtained from nitrogen-starved *nod*$^+$ or *nod* cultures, under inducing (i.e. presence of luteolin) or non-inducing (absence of luteolin) conditions (Fig.1). The peaks representing succinylation (methylene protons; a doublet at ≈2.6 ppm), acetylation (methyl proton; a singlet at 2.2 ppm) and pyruvylation (methyl; a singlet at 1.45 ppm) were present in spectra obtained from supernatants of both *nod*$^+$ (Rm1021) and *nod* (Rm5613) strains, whether or not inducer had been added to the cultures. The complex regions between 3.3 ppm and 4.9 ppm, representing the ring protons and β-anomeric protons of the sugar constituents, were difficult to compare, but no obvious differences were evident. The spectra obtained agree with the published spectrum for this EPS (Leigh et al., 1985; see also Aman et al., 1981 for detailed structural analysis).

Attempt at phenotypic complementation of *exo* mutants by EPS from wild type and *nod* cells: Seedlings were inoculated with Rm5078 (*exoB355*) followed by addition of concentrated supernatant from nitrogen-starved *nod*$^+$ or *nod* cultures. grown in the presence or absence of luteolin. Control seedlings were inoculated with supernatant alone or Rm5078 alone. There was no obvious effect of addition of supernatant to plants, with or without bacteria: in the absence of bacteria no nodules were formed, whereas in the presence of bacteria, typical *exo*-type nodules (small, white or brownish, Fix$^-$; Finan et al., 1985; Leigh et

al., 1985) were produced, whether or not supernatant had been added to the plants.

Fig. 1. ^{1}H-NMR spectra of dialyzed supernatants from *nod*$^{+}$ and *nod* *R. meliloti*. Growing cultures were treated with luteolin and starved in nitrogen-free medium for two days. Top panel: Rm1021 (wild type). Bottom panel: Rm5613 (*nodC*::Tn*5*). The singlet at 1.5 ppm represents pyruvylation, that at 2.1 acetylation. The doublet at 2.5 ppm represents succinylation. The complex regions from 3.3 to 4.0 and 4.5 to 4.9 ppm represent the ring protons and β-anomeric protons of the saccharide rings, respectively. Similar spectra were obtained in the absence of luteolin treatment.


(A) RM1021
(B) RM 5613
6.500
5.500
4.500
3.500
2.500
1.500
FREQUENCY (PPM)

DISCUSSION

We have obtained genetic evidence for an interaction of Nod and Exo functions in nodulation of alfalfa by *R. meliloti* (Chapter 3). The simplest interaction would involve a chemical modification of EPS which is controlled by Nod function. We therefore looked at EPS produced in *vitro* by *nod*$^+$ and *nod* cells, under conditions where *nod* is known to be induced (Peters et al., 1986). However, our data show that all the major acidic substituents of wild type EPS were produced under all conditions tested, and there were no obvious differences in the regions of the spectra corresponding to the sugar backbone of the polysaccharide. This does not rule out the possibility of chemical modification in *vivo* under the much more complex conditions that exist in the nodule. Further experiments might include adding whole plants to cultures during EPS overproduction, as the Nod-Exo interaction could also require interaction with the plant. If these experiments were productive, one could attempt to isolate active plant factors. Similarly, one could try better to imitate in *vivo* conditions by producing EPS in a microaerophilic environment. Moreover, since *de novo* protein synthesis may be required, it might be preferable to induce overproduction of EPS by growth in glutamate rather than by nitrogen-starvation (Zevenhuizen and van Neerven, 1983; Sue Long, pers. comm.).

We did not attempt to separate polysaccharide from secreted oligosaccharide, but rather obtained ^{1}H-NMR spectra of total dialyzed supernatants from N-starved cultures. Zevenhuisen and Van Neerven (1983) have shown that *R. meliloti* SU47 secrete an octasaccharide which has the structure of the repeating unit of the acidic EPS produced by that strain. It is not known whether the octasaccharide is an

intermediate in EPS biosynthesis or whether it is a product of EPS hydrolysis. It is also not known whether it is the octasaccharide or the polysaccharide, or some other related species, that is the active principle in nodulation.

Phenotypic restoration of Fix$^+$ phenotype by addition of exopolysaccharide and related oligosaccharides to plants infected with EPS-deficient mutants has been reported for *R. trifolii* (Djordjevic et al., 1987). In our laboratory, repeated attempts to correct the Fix$^-$ phenotype of *R. meliloti exo* mutants by addition of EPS or EPS-derived oligosaccharides upon inoculation of alfalfa have been unsuccessful. In the light of our genetic evidence (Chapter 3) for a difference in Exo function from *nod* versus *nod*$^+$ rhizobia we repeated these experiments using EPS from a *nod* mutant, but to no avail.

Thus, although the genetic evidence for an interaction of Nod and Exo functions is compelling, we have been unable to demonstrate a physical difference in EPS produced from *nod* versus wild type cells.

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APPENDIX 2: DEVELOPMENT OF A DIRECTIONAL, HIGH FREQUENCY OF MOBILIZATION SYSTEM FOR GENETIC MAPPING IN R. MELILOTI UTILIZING Tn5-*Mob*

INTRODUCTION: Several workers have devised genetic maps of the *R. meliloti* chromosome (Meade and Signer, 1977; Kondorosi et al., 1977; Kondorosi et al., 1980). These studies utilized the P-group plasmid RP4 (or its derivatives) which has the property of mobilizing chromosomal fragments, resulting in the formation of R-primes. By mating the R-prime into a suitable recipient, followed by selection for recombination, genetic loci can be mapped with respect to one another.

The RP4 mapping system has several drawbacks. Firstly, mobilization of any given locus occurs at low frequency (10^{-6} to 10^{-8}). Secondly, mobilization occurs from random (or nearly random) sites with equal frequency of transfer of markers equidistant from the origin of transfer in both directions.

We devised a strategy for genetic mapping which utilized directional, high-frequency mobilization of chromosomal fragments from Tn*5* chromosomal inserts at known locations. A derivative of Tn*5*, called Tn*5*-*Mob*, had been developed which incorporated the origin of transfer (*oriT*) of plasmid RP4, and it had been shown that *Rhizobium* megaplasmids could be mobilized *in trans* by RP4 at high frequency from Tn*5*-*Mob* inserts on those megaplasmids (Simon, 1984). A similar construct, Tn*5*-*oriT* (Yakobson and Guiney, 1984) has also been reported, and appears to have equivalent function to Tn*5*-*Mob*, although we found a lower frequency of transposition into the *R. meliloti* chromosome for Tn*5*-*oriT* than for Tn*5*-*Mob*. The transposition frequencies may have reflected the different

vectors on which the transposons were inserted or the differences in the Tn*5*-derivatives themselves; Tn*5*-*oriT* has a smaller RP4-derived fragment than Tn*5*-*Mob*.

<u>STRATEGY:</u> Tn*5*-*Mob* was linked by transduction to previously-mapped (Meade and Signer, 1977) auxotrophic markers located at intervals around the *R. meliloti* chromosome. The strains so constructed were mated with recipients which had auxotrophic markers on one or the other side of the Tn*5*-*Mob* in the donor, with selection for prototrophy. We found that:

a) Chromosomal segments could be mobilized from Tn*5*-*Mob* using either plasmid pRP4ΔK (pRP4 derivative sensitive to neomycin) in a biparental or triparental mating or pRK600 in a triparental mating (Finan et al., 1986). Since plasmid pRK600 does not replicate in *R. meliloti* whereas plasmid pRP4ΔK is very stable in *R. meliloti* the former was the preferred mobilizing plasmid ("helper") so that newly-constructed strains could serve as recipients in subsequent matings without further manipulations.

b) Transfer and recombination of loci known (by RP4 mapping) to be linked to the region in which the Tn*5*-*Mob* was inserted in the donor occurred at frequencies of up to 10^{-3}.

c) The frequency of recombination for any locus depended on its distance from the origin of transfer (i.e. from the Tn*5*-*Mob* insert in the donor).

d) Mobilization of chromosomal segments was directional. High frequency of recombinational rescue occurred only for markers on one side of the insert for any given Tn*5*-*Mob* insert. However, since Tn*5*-*Mob* inserted in

either orientation with approximately equal frequencies, it was possible to choose inserts which served as origins of transfer in either direction.

e) As a consequence of (a) through (d), genetic markers could be ordered by the relative frequencies at which they were mobilized and subsequently rescued by recombination using a given Tn*5*-*Mob* insert as the origin of transfer in a long (four hours to overnight) mating or in interrupted matings.

Thus, by choosing Tn*5*-*Mob* inserts in both orientations at each of three points in the circular chromosome, we could mobilize the entire chromosome (with few exceptions, see below) at relatively high frequency, thereby assigning any genetic locus an approximate location on a particular chromosomal segment. Although the system could presumably also be used for more precise mapping with two and three-factor crosses (as for Hfr mapping in *E. coli*), in *R. meliloti* SU47 derivatives we have utilized transductional mapping by ϕM12 (Finan et al., 1984) to further define the locations of genetic markers of interest.

Some loci might lie between linked Tn*5*-*Mob* inserts of opposite orientation and would thus "fall between the cracks" and fail to be mobilized by either origin of transfer. (This could be overcome by choosing Tn*5*-*Mob* inserts in such a manner as to obtain overlap of the initial sectors transferred or by using the insert-replacement system of De Vos et al. (1986) to choose origins in either orientation from an identical site. We have not yet precisely mapped the Tn*5*-*Mob* inserts and so do not know whether or not there is such overlap, and the method

of De Vos et al. was not yet available when this work was begun.) However, by definition any such loci must be transductionally linked to the Tn*5*-*Mob* inserts and to the auxotrophic marker to which those inserts are linked. Similarly, recombination of loci which are very tightly linked to the Tn*5*-*Mob* insert would not be detected if the segment in which a crossover must occur were too small. However, such markers would also be transductionally-linked to the Tn*5*-*Mob*.

DETAILS: *E. coli* S-17 (pSUP5011) (Simon, 1984) was mated with *R. meliloti* Rm1021 (SU47 *str-21*) with selection for neomycin resistance (Nm^r; 200 μg/ml) of Tn*5-Mob* and counterselection by streptomycin (Sm; 200 μg/ml). A lysate of phage ϕM12 (Finan et al., 1984) was grown on the pooled colonies containing Tn*5-Mob* inserts. This lysate was used to transduce a number of strains, including Rm3359 (SU47 *str-3 trp-33 his-39 pyr-49 nov-57 spc-1 rif-1*; H. Meade, PhD thesis, MIT 1977) and Rm3357 (*str-3 trp-33 his-39 leu-53 nov-57 spc-1 rif-1*; ibid.). Selection was for Nm^r plus prototrophy for individual nutrients, namely, leucine, tryptophan or pyrimidine on M9 medium (Maniatis et al., 1982) containing Nm and appropriate supplements.

Tn*5-Mob* inserts linked to a number of chromosomal markers were screened, but our results indicate that the following inserts are sufficient to mobilize the entire *R. meliloti* chromosome:

Insert #	Selected by linkage to	High-frequency transfer of*
601	*leu-53*	*trp-99, met-1023, ilv-13, cys-11*
602	*leu-53*	*ilv-48, trp-33*
612	*trp-33*	(did not transfer *met-56*)
611	*trp-33*	*aro-51, met-56, pyr-501*
615	*pyr-39*	*pyr-501*
614	*pyr-39*	*cys-11*

*High-frequency transfer was at 10^{-3} to 10^{-6}, with lower frequencies reflecting greater distance of the selected marker from the Tn*5-Mob* insert.

The utility of the system was demonstrated by locating the *trp-505* allele, which was mobilized by insert 601 at a frequency of 1.1×10^{-6} and by insert 637 (linked to *cys-11*) at a frequency of 9.8 x 10-5. Another *trp* allele, *trp-501*, which is in the same complementation group as *trp-99* (Martin and Long, 1984) and presumably maps in the same position, was transferred by insert 601 at a frequency of 2.6×10^{-5} and by insert 637 at a frequency of 1×10^{-5}. This suggests the following order: *leu-53, trp-501, trp-505, cys-11*.

In other experiments, inserts of Tn*5-Mob* linked to the *nod* region on megaplasmid pRmeSU47a were used in an attempt to map several *exo* loci. In each case, no transfer of *exo* was detected. We now know (Finan et al., 1986) that the *exo* loci concerned are either on pRmeSu47b (*exoA, B, F, E*) or on the chromosome (*exoC*).

FURTHER PLANS: In order to eliminate the necessity to construct new strains each time a locus is to be mapped, and for those cases where only screening and not selection is available, I plan to insert a selectable marker near the end of each chromosomal segment. I will develop one set of donor strains with the selectable marker Tn*5-233* which encodes resistance to spectinomycin (De Vos et al., 1986) and one set with the selectable marker Tn*5-Tp* which encodes resistance to trimethoprim (Appendix 5). The backgrounds for the various strains will be Rm3357 and Rm3359 (H. Meade, PhD thesis, MIT 1977) which contain multiple auxotrophies that can be used in counterselection.

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APPENDIX 3: CONSTRUCTION OF ϕM12H1, A BROAD HOST RANGE DERIVATIVE OF THE TRANSDUCING PHAGE ϕM12

ϕM12 is a transducing phage which we have used extensively for genetic manipulations (Finan et al. (1984), J. Bacteriol. 159, 120-124). I developed ϕM12h1 in an attempt to produce a phage which could be used for transducing DNA between *R. meliloti* SU47 and Rm41 derivatives.

An early logarithmic culture in LB (2.5 mM Mg^{++}, Ca^{++}) of a *R. meliloti* Rm1021 derivative was infected with ϕM12 and allowed to stand for 25 minutes at 30C. At this point, 1/5th volume of a 1:8 aqueous dilution of EMS was added to the lysate, and the tube was gently shaken. After two hours of incubation without shaking at 30C, several drops of chloroform were added to the lysate, which was harvested and titred as usual for ϕM12, and stored at 4C. The lysate was used to infect the Rm1021 derivative and the infected culture was incubated with shaking for 2 hours at 30C, at which point the infected culture was mixed with a 16-fold excess of an early exponential culture of AK631, allowed to adsorb for 20 minutes at 30C, without shaking, and plated as usual, in soft agar (supplemented with Mg^{++}, Ca^{++}). Clear plaques were picked and purified on AK631 indicator. The best mutant, although it gave clear plaques on AK631, still gave only low titre lysates, and was put through a second cycle EMS mutagenesis, as follows: A lysate was grown in λ (Mg^{++} and Ca^{++}) broth. The titre was about 5×10^8 pfu/ml. About 10^6 pfu were used to infect a growing culture of AK631. After allowing 20 minutes' adsorption at 30C, the infected bacteria were plated in soft agar (supplemented with Mg^{++} and Ca^{++}) on fresh plates of λ-agar (Mg^{++}, Ca^{++}) containing EMS at concentrations varying from 0 to 1%. After two

days' incubation, large plaques were picked from the plates with EMS at 0.05 and 0.1% and purified. The best mutant obtained was named ϕM12h1.

ϕM12h1 gave clear plaques on both AK631 and Rm1021 derivatives, and was as competent as ϕM12 for transduction between SU47 derivatives. Although titres of up to 5 x 10^9 were obtained on Rm6902 (=AK631*met*::Tn*5*), few or no Nm^r transductants were obtained in either Rm1021 or AK631. Nonetheless, the mutant was used to transduce *exoA* and *exoF* markers from Rm1021-derivatives into AK631. A mutant of Rm1021 with altered LPS (Rm7520) which is insensitive to ϕM12 was found to be sensitive to ϕM12h1 which was used to characterize the LPS-mutant genetically (Ralph Clover, pers. comm.).

APPENDIX 4: CONSTRUCTION OF A RECOMBINANT DNA LIBRARY FROM *RHIZOBIUM MELILOTI* RM41

A library of genomic DNA from *R. meliloti* Rm41 in plasmid pLAFR3 (B. Staskowicz, unpub.) was prepared as follows. Rm41 genomic DNA was prepared by the method of Marmur (1961) as adapted by Gary Barsomian (pers. comm.). The DNA was partially digested with restriction endonuclease *Sau*3AI. The DNA was run over a sucrose gradient (Maniatis et al., 1982) and the fractions with fragments in the size range 15-25 kb were pooled.

Plasmid pLAFR3 was derived from plasmid pLAFR1, a broad-host-range cosmid cloning vector (Friedman et al., 1982) by inserting a polylinker in the *Eco*RI site of that plasmid (B. Staskowicz, pers. comm.). Plasmid pLAFR3 DNA was restricted with either *Eco*RI or *Hin*dIII followed by treatment with calf intestine phosphatase and restriction with *Bam*HI. Both of the pLAFR3 "arms" resulting from this treatment were mixed with the partially-digested genomic DNA and ligated. The ligation mixture was packaged using the Gigapack Gold λ packaging mix of Stratagene, Inc. according to the instructions in the kit. The packaged DNA was transfected into *E. coli* S17-1 (Simon et al., 1982), with selection for resistance to tetracycline (on LB plus tetracycline, 10 μg/ml).

Individual colonies were picked into LB broth containing tetracycline in 21 microtitre plates, leaving several blank wells on each plate as controls for contamination. After growth at 37°C, followed by replication, DMSO was added to each well to a final concentration of 10% and the library was stored at -70°C. Assuming an insert size of 20 kb and total genomic size of 4000 kb, there is a

probability for complete coverage of the genome of greater than 99.9% (Maniatis et al., 1982). The library was also amplified and stored in aliquots of 300 Ml; each aliquot was estimated to include 3,800 primary clones which gives a similar probability of including every gene.

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APPENDIX 5: CONSTRUCTION OF A DERIVATIVE OF TN5, TN5-TP, ENCODING RESISTANCE TO TRIMETHOPRIM

The only transposon which is known to transpose randomly into *R. meliloti* is Tn*5*, which encodes resistance to neomycin, bleomycin and streptomycin (Berg et al., 1975; Genilloud et al., 1985; Mazodier et al., 1982). A number of derivatives of Tn*5* have been developed, encoding different antibiotic resistances (De Vos et al., 1986). I constructed Tn*5*-*Tp*, which incorporates the trimethoprim-resistance fragment of Tn*402* (Meyer and Shapiro, 1980), as follows.

Plasmid pWB6 (W. Buikema, unpub.) yields two fragments upon digestion with restriction endonuclease *Bam*HI, of 4.4 and 2.7 kb. The 2.7 kb fragment, which encodes resistance to trimethoprim, was used to replace the central *Bgl*II-*Bgl*II fragment of a Tn*5* insert in plasmid pGS330 (De Vos et al.,1986). Plasmid pWB6 was completely digested with *Bam*HI, plasmid pGS330 containing a Tn*5* insert was completely digested with *Bgl*II, and the reactions were terminated by ethanol precipitation of the DNA. The digests were mixed and ligated, followed by *Bam*HI digestion to restrict any religated pWB6, and the DNA was transformed into *E. coli* MT609 (*polA1 thy* Sp^r; T. M. Finan, unpub.), with selection for resistance to trimethoprim (25 μg/ml) on M9 agar (supplemented with glucose, thiamine and casamino acids). *Hin*dIII digestion of DNA from transformants yielded three fragments of 4.7, 2.3 and .6 kb, as expected.

One transformant was chosen for further work, and the DNA therefrom was transformed into *E. coli* MT607 (*pro-82 thi-1 hasdR17 supE44 recA56*; Finan et al., 1986). *E. coli* MT607 (pGS330 with Tn*5*-*Tp*

insert) was mated with *R. meliloti* Rm6022 [SU47 *rif-5 recA*::Tn*5* (pRK290.34 *recA*$^{+}$); G. De Vos, pers. comm.; De Vos et al., 1986; Better and Helinski, 1983]] in a triparental mating using plasmid pRK600 (Finan et al., 1986) to mobilize the pGS330 derivative. The mating mix was plated on LB agar supplemented with trimethoprim (650 μg/ml) and rifampicin (50 μg/ml). Counterselection on minimal medium (M9) was also attempted, but the rifampicin counterselection gave less background. On the rich medium, colonies arose at a frequency of approximately 2 x 10^{-5} per recipient. Out of 300 colonies tested, one was sensitive to neomycin, indicating a double recombination event resulting in replacement of the Tn*5* in Rm6022. A ϕM12 lysate was grown on the neomycin-sensitive colony and transduced into Rm1021 (SU47 *str-21*) and Rm5000 (SU47 *rif-5*) followed by plating on LB agar containing trimethoprim (650 μg/ml). The colonies so obtained were sensitive to UV.

E. coli MT607 (pGS330 with Tn*5-Tp* insert) was also mated with Rm1021, in a triparental mating utilizing pRK600 as mobilizing plasmid, with selection for resistance to trimethoprim and streptomycin. The frequency of transposition was approximately 2 x 10^{-6} per recipient. The colonies obtained were replicated onto minimal medium and two proved to be auxotrophs, one requiring leucine and the other uracil or arginine (*pyrA*?), indicating random transposition in *R. meliloti* by Tn*5-Tp*.

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