The genetic organization of nitrogen-fixing rhizobium species: past and future applications to agriculture.

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Importance of the legume-rhizobium symbiosis to agriculture

Nitrogen is an integral component of many biologically important organic molecules. Consequently, the growth and productivity of important non-leguminous agricultural species is largely dependent on the availability of soil nitrogen. Australian soils however, in their natural state are notoriously deficient in nitrogen (1). The benefits derived from applying nitrogenous fertilizers are easily recognized in terms of plant productivity (i.e., yield). But due to the high cost of nitrogen-based fertilizers and their application, the use of nitrogen fertilizers for forage species and cereal crops is generally uneconomical.

Through a symbiotic association with a soil bacterium (Rhizobium), leguminous plants (e.g. clovers, medics, etc) have the unique ability to utilize (fix) atmospheric dinitrogen gas. Rhizobia (Rhizobium sps) are able to induce the formation of morphologically defined structures (nodules) on the roots of members of the plant family Leguminosae. It is within these nodules that the bacterium is able to reduce atmospheric nitrogen into ammonia, which is subsequently available to the host legume for assimilation into organic molecules for growth and plant function. Accumulated nitrogen in legumes can be made available to other desirable agricultural species (including non-leguminous plants) upon senescence and decomposition of the host legume plant (Figure 1). By this means, nitrogen fixed by the legume-Rhizobium association can be utilized as a nitrogen source for subsequent cereal crops grown in rotation with legumes, or to provide nitrogen to grasses growing in mixed pasture swards. Indeed, because of the widespread nitrogen deficiency of Australian soils and the high cost of applied nitrogen, the nodulated legume has had a distinct role in the development of improved pastures and improving the nitrogen status of soils throughout vast areas of Australia (1). It has been estimated that nitrogen fixed in Australia by subterranean clover alone, is worth more than 650 million dollars annually (2).

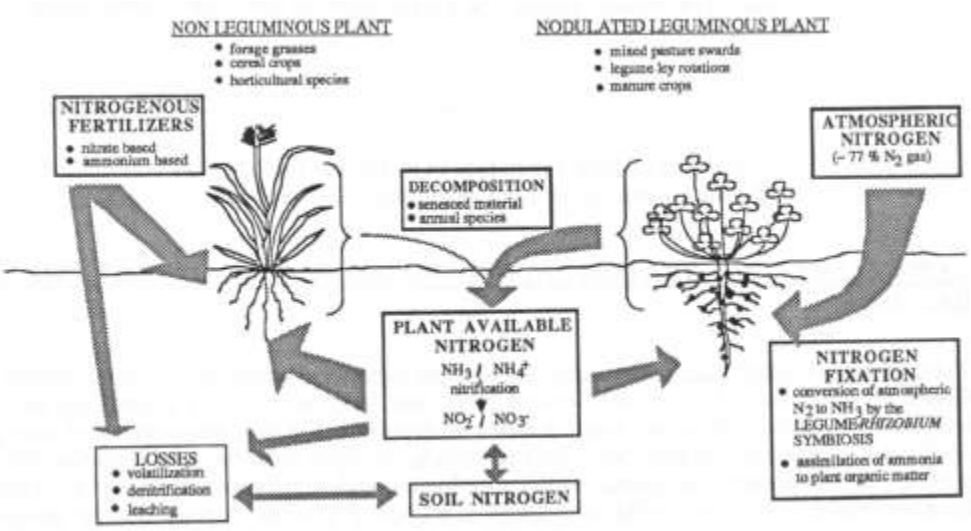


Figure 1. Cycling of nitrogen in soils. Nitrogen fixed symbiotically by nodulated legumes markedly contributes to the plant available nitrogen pool and consequently reduces the need for nitrogenous fertilizers.

Inoculant strains of rhizobium

To capitalize on the enormous benefits derived from symbiotic nitrogen fixation, Australia has developed a highly successful inoculant industry. The Australian Inoculants Research and Control Service presently ensures that high quality inoculants are maintained and made available to inoculant companies for distribution throughout Australia (3). A continuing problem however, is to maintain inoculum strains true to their original phenotypic properties and reported declines in the performance of inoculum strains is cause for concern(4). Thus, selection and testing of specific strains for use as inoculants is an ongoing requirement and to the present has relied on selections from naturally occurring populations of Rhizobium collected from specific localized regions. Selected strains must be; competitive with indigenous rhizobia for nodule initiation and formation, must nodulate promptly and at a high level of effectiveness over a range of environments, and must be persistent in the soil for several years after their introduction (5). Despite research efforts over several decades which has resulted in many successful inoculants and because of the ongoing requirement for "better" inoculants, potential still exists for further gains to be made in the development of specific inoculant strains (e.g. Table 1). Recent advances in molecular biology has rapidly enhanced our knowledge of the complex interactions between legumes and their symbionts. The advent of newly developed molecular techniques provides us with great potential to "modify" the legume-Rhizobium symbiosis.

Table 1. Some areas where techniques in molecular biology may contribute to the improvement of inoculant strains of Rhizobium.

1.	Increased stability of the symbiotic capacity (effectiveness) of *Rhizobium* strains.
2.	Enhancement of saphrophytic, parasitic and symbiotic competence of inoculum strains. Their ability to successfully nodulate hosts when in competition with indigenous strains in soil environments.
3.	Development of persistent and effective (nitrogen-fixing) inoculant strains for different soil types and under a variety of environmental conditions. -acid soils -saline soils -temperature / moisture extremes in soil -soils with mineral deficiencies and toxicities -soils with high nitrate
4.	Selection of suitable strains that are effective on newly developed and/or alternative legume species.

Rationale behind the push to understand the biology of rhizobium bacteria

The past two decades have seen drastic improvements in food production in part because of the Green Revolution. The benefits derived from this "revolution" only extend the time until increased population pressures cause a renewed problem. Genetic engineering of crop plants (including nitrogen-fixing legumes) could potentially increase this time span until the population pressure on our planet is in balance with its biological and environmental resources. However, because yield is polygenically controlled (i.e., many genes), it may not be possible to substantially increase the yield component of plants through in vitro genetic engineering techniques. Polygenic attributes at the present time, can only be manipulated by using standard plant breeding techniques. In contrast, we can manipulate the soil bacterium Rhizobium. If we consider that the major costs in biological production are (a) fertilizer use; (b)

use of pesticides and herbicides; and (c) the cost of application of these products. The importance of Rhizobium and legumes is that first, their symbiotic association reduces the need for nitrogenous fertilizer and secondly, the costs associated with the use of fertilizers, pesticides and herbicides could be lowered by the use of alternative biological means. These two factors constitute an important contribution to modern agriculture.

It is our view that an important contribution to this overall problem is to rapidly generate a large body of fundamental information on biological nitrogen fixation, particularly with respect to the symbiosis between Rhizobium bacteria and legume plants. Such information can then be readily applied to the improvement of the growth and development of both forage and grain legume crops.

Genetic organization of nitrogen-fixing Rhizobium species.

Rhizobium species

Rhizobium species are defined on the basis of their host-range characteristics. Particular legumes are nodulated only by certain Rhizobium strains. For example, R.trifolii nodulate clovers (Trifolium species), R.leguminosarum nodulate peas, (Pisum sativum), R.meliloti nodulate lucerne (Medicago saliva) and slow-growing rhizobia, Bradyrhizobium japonicum nodulate soybeans (Glycine max). The rhizobia which invade temperate legumes tend to exhibit a narrow plant host-range (6). In contrast, Rhizobium strains that infect tropical legumes tend to be less restricted and can invade a broader range of these plants.

The original classification of rhizobia into two distinct groups ("fast-growers" and "slow-growers") based on their physiological and growth characteristics (6), has recently been replaced with classification into two genera: the fast-growing Rhizobium species and the slow-growing Bradyrhizobium bacteria.

Rhizobium occupies two habitats

The soil bacterium Rhizobium has two distinct life styles, one within the soil, the other within the induced root nodule of an infected host. Different genes are required for dealing with these two habitats. A phenotypic shift occurs which results in the transition of the rhizobia from being a free-living "saprophyte" in the soil and rhizosphere, to one of being a "parasite" on the root surface, to eventually becoming a "symbiont" within the plant tissue (7) (Figure 2). The rhizosphere and mucigel layer are rich in growth-stimulating substances (8) which may facilitate the attraction of rhizobia to the plant root surface from the surrounding soil environment. Rhizobia colonize the whole root surface and attach to epidermal and root hair cells (Figure 3). The majority of these bacteria do not initiate infections of the plant cells but remain associated either on the root surface or in the mucigel layer. Some, however, specifically interact with newly emerging root hairs and initiate a pronounced curling of these growing hair cells. To initiate this interaction, rhizobia have adapted to use flavonoid compounds released by the plant into the root rhizosphere to induce their infection (nodulation, nod) genes. Following the initiation of an infection process, rhizobia entrapped within curled root hair cells begin the invasion of these plant cells. Invasion occurs via the induction of an infection thread which penetrates the plant tissue and continues to grow and ramify in the root cortex.

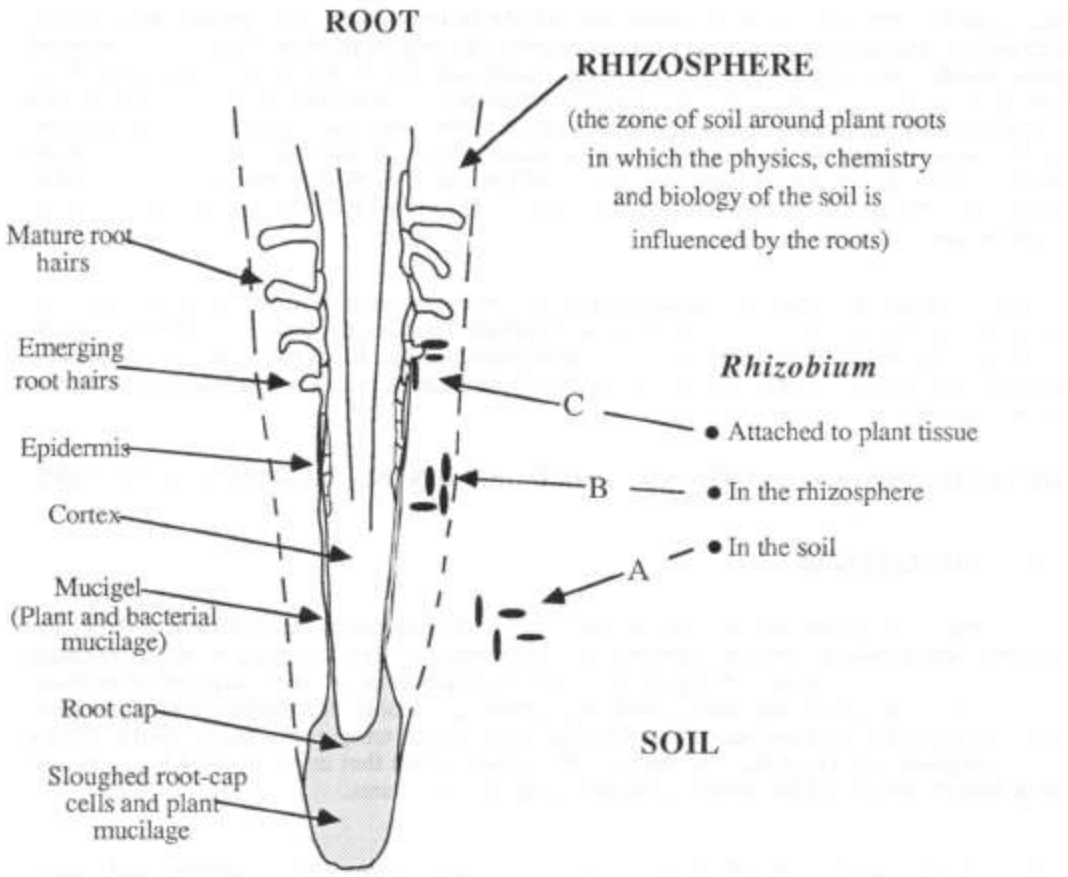


Figure 2. Diagram of a typical legume root. Rhizobia exist either as; saphrophytes in the soil and rhizosphere or as parasites on the root surface before becoming symbionts within the plant tissue.

During the early stages of infection thread formation a Rhizobium-derived signal is thought to cause the induction of some of the cortical cells to divide (Figure 3). The infection thread eventually invades this focus of dividing plant cells and rhizobia are released into these cells after they are first "packaged" within a plant membrane. The bacteria continue to grow and ultimately differentiate into bacteroids which are capable of fixing nitrogen (Figure 3). A specific signal from the plant is thought to stimulate the derepression of the nitrogenase and other genes in the bacterium involved in nitrogen fixation (6). These changes in microbial properties requires that many of the genes involved in the saprophytic mode must become repressed and genes involved in plant colonization, infection and invasion leading to a nitrogen fixing nodule be induced and coordinated in their activities.

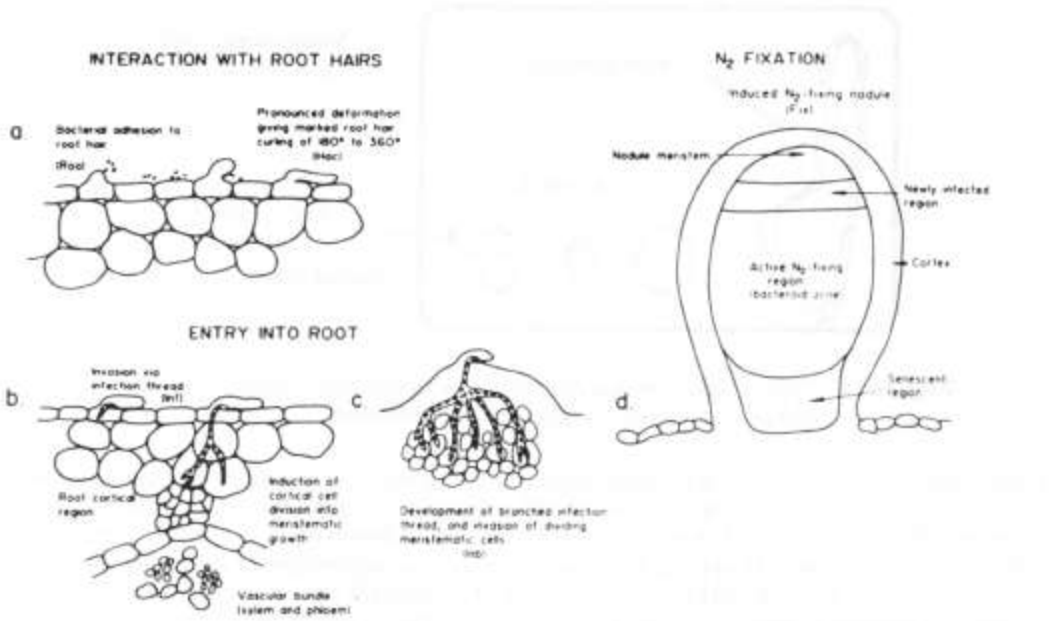


Figure 3. The sequence of events in the development of a clover root nodule

Importance of plasmids to fast-growing rhizodium strains

Nearly all of the fast-growing Rhizobium strains have been shown to possess large plasmids which are circular DNA elements that can replicate independently of the chromosome. The number and size of these plasmids varies from strain to strain and often differs in properties such as incompatibility, self-transmissibility and bacteriocin production (9). The genetic determinants responsible for the ability of fast-growing Rhizobium to recognize a plant host, to invade, to induce nodule formation and to fix atmospheric nitrogen, have been shown to be located on a large indigenous plasmid (Symbiotic of Sym plasmid, Figure 4) (10,11). Most fast-growing Rhizobium strains carry only one Sym plasmid. Sym plasmids can be removed (cured) from many strains and the resulting mutant cells ($pSym^-$) are unable to interact with their respective legume hosts.

The use of transmissible Sym plasmids has been useful in examining the interaction of these plasmids and the contribution made by the background genome of the bacterial cell to the phenomenon of host-specificity. For instance, when a resident Sym plasmid is cured ($pSym^-$ strain) from a R. trifolii strain, the ability to nodule the host plant (clovers) is lost. The introduction of a Sym plasmid from another Rhizobium species, for example R. legunzinosarum, results in a strain which has the host-range properties of R. leguminosarum (12,13). This reconstructed strain fails to nodulate clovers (its original host) but can now nodulate pea plants. If instead an R. trifolii Sym plasmid was introduced to this $pSym^-$ strain the original host-range properties were restored.

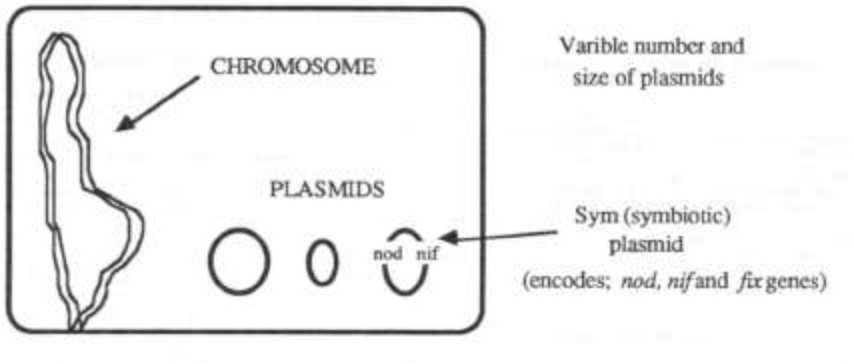


Figure 4. Typical fast growing Rhizobium with symbiotic genes (nod, *nif* and fix) on a large indigenous mega-plasmid.

While nodulation properties of Rhizobium strains are primarily determined by the Sym plasmid, the background of the cell plays a role in influencing the expression of a nitrogen-fixing response. The Sym plasmid is known to carry many genes involved in conferring a nitrogen-fixing (Fix+) response. However, because the effective expression of fix genes does not always occur in a foreign background cell (14), it is thought that a contribution from the background genome is required for the Fix + response. This is particularly evident when Sym plasmids were transferred to the closely related soil pathogen Agrobacterium tumefaciens (which induces galls on dicotyledonous plants). Transfer of Sym plasmids to this strain resulted in the transfer of nodulation ability but nitrogen-fixing nodules were not produced (12,15,16,17).

The genes required for metabolic pathways, energy production, electron transport, metabolite uptake systems, polysaccharide synthesis and some genes affecting the nitrogen-fixation phenotype have been shown to be located on the Rhizobium chromosome (9,18,19). The arrangement of genes on the chromosomes of several fast-growing Rhizobium species and the soil microbe Agrobacterium tumefaciens have also been shown to be very similar (18).

Molecular techniques for the genetic analysis of rhizobium strains

Efficient plant screening procedures and mutagenesis systems have been recently developed for studying the clover-Rhizobium symbiosis (20,21). These have involved the use of transposons which are small elements of DNA that can insert into different regions of the genome of a bacterium. Transposons usually carry a drug resistance gene which can be used to show the location of insertion into the bacterial DNA (22). The "jumping" of a transposon into a particular gene causes an insertion mutation as the target gene sequence is interrupted by the insertion event. This then provides a 'tag' for particular genes of interest. The transposon Tn5, (which carries a kanamycin resistance marker), has been particularly useful as it has minimal site preference for insertion and has no restriction enzyme sites for EcoRI, ClaI and KpnI endonucleases. It can therefore be readily used in molecular cloning experiments (22).

In the Rhizobium bacterium, genes of interest have been located by transposon mutagenesis by screening for the loss of function of the trait of interest. Current molecular techniques (cloning) have been used to isolate the transposon and associated Rhizobium DNA sequences (23,24). The coupling of these techniques with a procedure for rapidly screening bacterial mutants for symbiotically-defective associations with plants (20) provides a system for the isolation of mutants in the pathways involved in establishing a nitrogen-fixing nodule.

Although identified, the precise functions of the various nodulation (*nod*) genes and their regulation have essentially remained unknown. This is largely because attempts at isolating nod gene mRNA and corresponding protein products from free-living Rhizobium cells have been unsuccessful (25). Protein products of the *nod* genes have been obtained only by fusion of the genes to strong Escherichia coli

promoters (26) or by using an E. coli in vitro transcription / translation system, whereby gene products corresponding to specific genes are produced (e.g. *nod*C and *nod*D) (27). Thus, another powerful genetic technique for analyzing bacterial genes has been to construct Rhizobium strains with the lacZ (B-galactosidase) gene from E. coli fused to specific genes of interest. This procedure has been used successfully for analyzing nodulation (*nod*), nitrogenase (*nif*) and nitrogen fixation (fix) genes (28,29,30,31,32). The use of B-galactosidase fusions has enabled the identification of different complementation groups; determination of the direction of gene transcription; identification of the location of promoter genes and ribosomal binding sites; and lead to the isolation and identification of compounds released in the exudates from plants which are responsible for the activation of the Rhizobium *nod* genes (33,34,35,36).

Genetic organization of the nodulation (*nod*) genes

The use of the above techniques has enabled the location of the nodulation (*nod*) genes to be determined in several fast-growing Rhizobium. Analysis of Tn5 induced non-nodulating (Nod-) mutants identified a set of four contiguous and highly conserved genes (Figure 5), designated *nod*DABC (25,37,38). In R. trifolii and R. leguminosarum, mutations in these genes resulted in strains that were unable to curl root hairs (Hac- phenotype) of their respective hosts.(27,38,39,40). In R. meliloti, *nod*ABC mutants have also been shown to be Hac(41,42). However, because in this strain the *nod*D gene is reiterated on the Sym plasmid, *nod*D mutants retained infection ability but the nodulation response was less efficient and delayed

DNA sequence analysis has shown that the *nod*D gene is linked to the *nod*ABC genes but,is transcribed divergently. The *nod*ABC genes have been shown to be part of a single operon (Figure 5). Furthermore, interspecies complementation studies have shown that the *nod*DABC genes are functionally interchangeable between species (25,44) and thus have been called the "common" *nod* genes.

Two genes, *nod*I and *nod*J , have been identified downstream of the *nod*ABC operon in several Rhizobium strains (Figure 5) and are involved but are not obligatory for nodulation in some rhizobia (44,45). Mutation of these genes in different Rhizobium species resulted in mutants with varying degrees of disability. In R. trifolii , mutations in *nod*I and *nod*J resulted in enhanced root hair curling (Hac++) but, induced only a few poorly formed nodules on clovers. Depending on the Rhizobium species and the legume host involved, the *nod*IJ genes appear to play a role in determining host-range properties of strains. .In R. leguminosarum, *nod*IJ mutants are Nod- on the common vetches while being delayed in their nodulation of peas (Wijffelman, pers. comm.).These two genes (*nod*JJ) probably are part of the *nod*ABC operon, which is regulated through the *nod*A promoter (Figure 5).

A third region of genes in R. trifolii involved in legume nodulation are the *nod*FELM cluster of genes (Figure 5). Mutations in the *nod*FE genes give a hyper root hair curling (Fin++) response and an alteration of R. trifolii host range. While the wild type strain is able to extensively and rapidly nodulate white, red and subterranean clovers, *nod*FE mutants show a distinct inability to nodulate white and red clover species. The nodulation response of *nod*FE mutants on subterranean clovers is only subtly affected. The mutant strains, in contrast to the parent strain, form infection threads in and nodulate both European and Afghanistan peas. These mutants also induce infection threads on white clovers but fail to initiate nodule formation

This indicates that (a) the *nod*FE genes are involved in the conferral of host range, (b) they are dominant to other host range genes, (c) white clover plants can prevent the mutants from nodulating, and (d) pea plants can prevent the wild-type strain (but not the mutants) from nodulating. Moreover, it has been shown that these genes involved in host-range ability are not functionally conserved between different Rhizobium strains (25,46). The newly identified gene *nod*O (J. M. Watson, pers. comm.) also appears to be a host-range gene required for nodulation of Afghanistan peas (Wijffelman, Rolfe, Djordjevic, unpublished). The hsn genes *nod*LM are intimately involved in root hair curling and the initiation of infection threads (Djordjevic et al., unpublished).

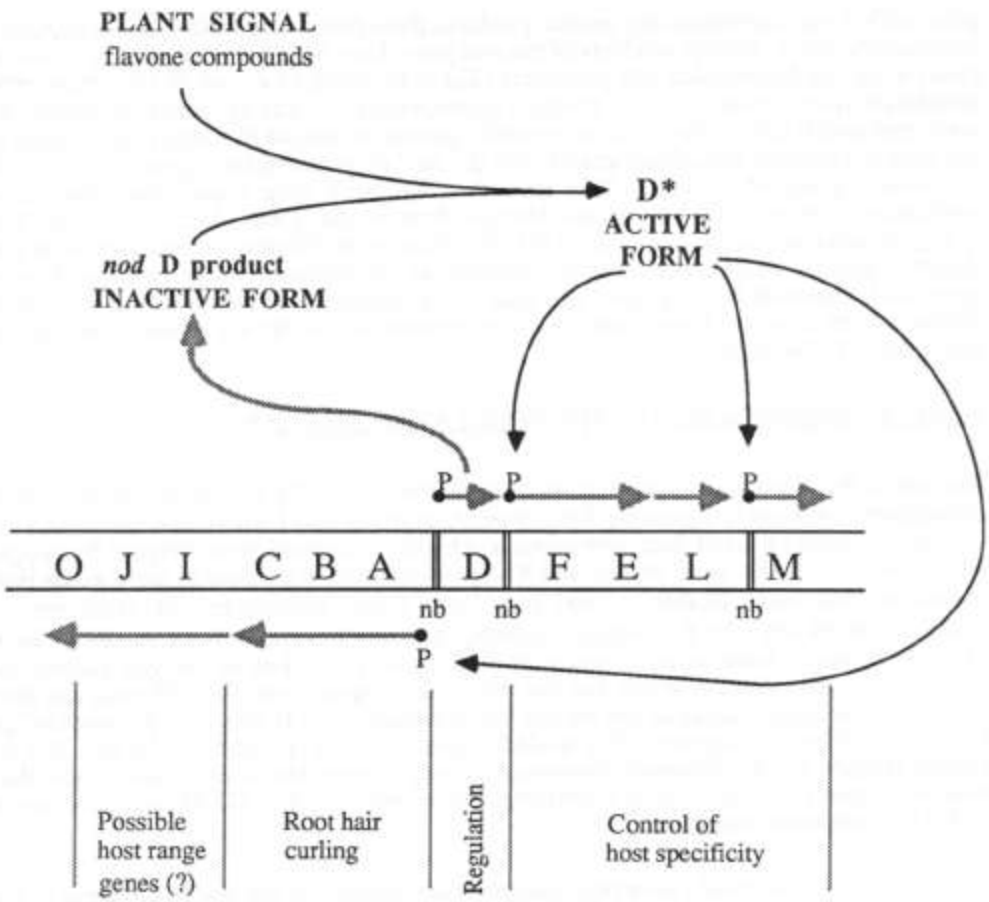
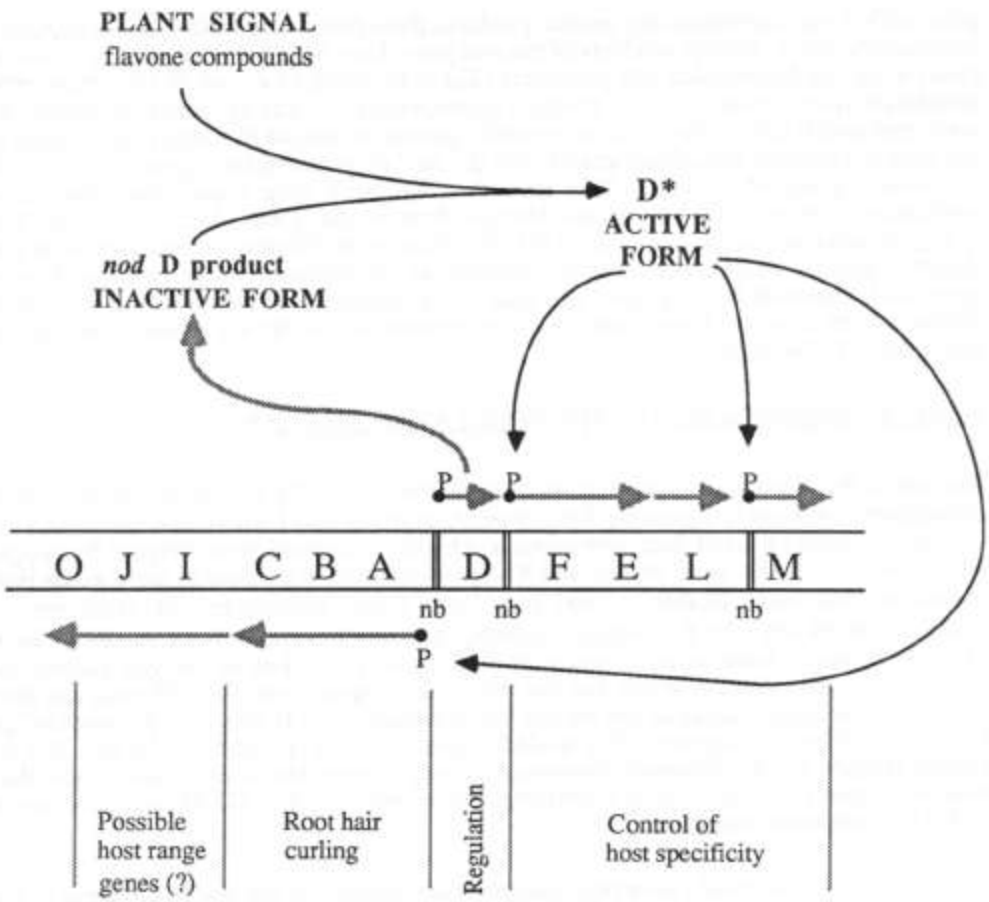


Figure 5. Organization of nodulation (*nod*) genes in Rhizobium trifolii and a proposed model of their regulation.

Also shown on Figure 5 is a reiterated DNA sequence of about 26 to 28 bases called the "*nod* -box" sequences (nb) which form at least part of the promoters of several flavonoid-induced *nod* gene operons (e.g. *nod*ABC, *nod*FE). This DNA sequence has been found to be common in both the fast and slow-growing rhizobia. Six such *nod*-boxes occur in R. meliloti (47) and several occur 5' to inducible (*nod*) operons in R. leguminosarum and R. trifolii (48,49). The *nod*D product is thought to interact with the *nod*-box sequences to promote transcription of the *nod* genes downstream from these *nod*-box sequences in a coordinated manner (47,48).

Regulation of the nodulation genes of rhizobium species

The development of an assayable system (Rhizobium strains containing lacZ genes from E. coli fused to *nod* genes) for the detection of plant derived stimulatory compounds has enabled the isolation and identification of the compounds which are responsible for the activation of *nod* genes. Stimulatory compounds have now been isolated from clovers, 7,4'-dihydroxyflavone (DHF) (33); and from alfalfa, luteolin (34). For peas, apigenin-7-0-glucoside and eriodictyl have been suggested as the chief stimulatory compounds (35). These stimulatory compounds are derived from branch points in the plant phytoalexin producing pathway (50) and are active at low concentrations (5 x 10-7 M DHF can give up to a 25 fold increase in the level of transcription of a R. trifolii *nod* A: :lacZ fusion). For the tropical legume soybean, the isoflavones daidzein and genistein and coumestrol have been isolated and shown to stimulate Rhizobium *nod*::lac Z fusions (B.J. Bassam et al. in prep.).



These stimulatory hydroxyflavones and isoflavones, which are released only from the zone of emerging root hairs, have been shown to rapidly induce bacterial *nod* genes (33,34,35,36). Whereas, the common breakdown products of the flavonoid compounds (hydroxybenzoic acids) have no stimulatory affects. Recent studies have also identified compounds secreted by legumes which inhibit the induction of *nod* gene fusions. Two compounds (umbelliferone, a coumarin; and formononetin, an isoflavone) have been identified in white clover exudates (36). The inhibitory mechanism appears to be competitive. The release of these compounds varies over the developing clover root, as does the production of the stimulator compounds. While the stimulator is chiefly released behind the growing root tip in the zone of emerging root hairs, the release of the inhibitor occurs in the region between this zone and the root tip and elsewhere on the root (36). The target for the action of the inhibitory and stimulatory compounds is either the *nod*D product or a common target which is likely to be membrane-bound. In vitro experiments have shown that the activation of *nod* genes is determined by the ratio of stimulator to inhibitor. As this ratio varies over the developing root, the earlier observation that nodule initiation is largely confined to a narrow window in the zone of emerging root hairs (51) takes on new significance. It is proposed that the stimulator:inhibitor (S:I) ratio is the chief determinant of the sites of nodule initiation. Fluctuations in the concentrations of the stimulatory and inhibitory compounds in the plant may also affect the expression of the *nod* genes in the infection thread. An excess of inhibitory compounds may cause the infection thread to cease because the bacteria are not able to continually stimulate the plant to either continue synthesis of the infection thread cell wall or continue cortical cell division (Figure 3). Regulation of flavone, coumarin and isoflavone synthesis may also be a mechanism by which legumes prevent over-nodulation. The inhibitory compounds could be the rapidly-induced systemic factor(s) which control nodule number (52,53,54).

Recent findings of ours suggest that the *nod*D (regulatory) gene product is probably a type of "environmental sieve", sensing the concentrations of stimulatory and inhibitory compounds released by a particular legume (36,55). Some species of Rhizobium contain multiple copies of the *nod*D gene which suggests that specific *nod*D-flavone interactions may occur (56,57,58,59). The expression of the R.trifolii *nod*D gene has been shown to be constitutive, but has a distinct peak in early log phase cells. Moreover, the flavone DHF is not required for the expression of the *nod*D gene (36). The current working hypothesis is that the *nod*D product is regulatory and requires the presence of the plant stimulatory compound to convert it to an active form which is involved in the initiation of expression of the *nod*ABC and *nod*FE operons and probably the expression of the other inducible *nod* genes (Figure 5).

The nodulation genes of the slow-growing bradyrhizobium species

The genetic analysis of slow-growing rhizobia has tended to be less advanced when compared to the fast-growing rhizobia. Nodulation mutants have been isolated and various *nod* genes (*nod*DABC and *nod*IJ) have been identified and located on the bacterial chromosome of the slow-growing Bradyrhizobium bacteria (60,61,62). A novel gene (*nod*K) of unknown function, occurs between the *nod*D and *nod*ABC operons in several of the bradyrhizobia (62,63). Genes homologous to the host specificity (hsn)-like sequences of strain MPIK3030 (a fast growing-broad host range Rhizobium) which confer siratro nodulation have also been identified (63,64). Mutation of the corresponding genes in B. japonicum strain USDA110 results in the loss of the ability to nodulate siratro but does not detectably affect soybean nodulation. The results to date indicate that different sets of host specific nodulation genes (or hsn "cassettes") may occur in these strains and this feature may be common in several Rhizobium and Bradyrhizobium species which show an extensive host range ability.

Genetic organization of the nitrogen fixation (*nif*, fix) genes

The two key regions necessary for the establishment of a nitrogen fixing nodule (*nod*, genes for the invasion and induction of a root nodule and *nif*, genes for the structural components of the nitrogenase enzyme complex) have been located some 16-30 kilobases (kb) apart on the Sym plasmids of several different fast-growing rhizobia (Figure 4). A detailed analysis of one particular R. trifolii strain (ANU843), has located the *nif* and *nod* gene clusters at approximately 16 kb apart on the 180 kb Sym plasmid (65).

Three nitrogenase structural genes have been identified; the *nif*H gene encoding the Fe-protein component of the nitrogenase enzyme complex and the *nif*DK gene encoding the Mo, Fe-protein sub-units. These genes are closely linked and probably form part of the same operon.(Figure 6) (66,67). The R. trifolii Fe-protein (*nif*H gene product), as predicted from the DNA sequence, is 297 amino acids in length and has a molecular weight of 31,903 daltons (66). It is now well established that the amino acid sequence of the Fe-protein subunit is strongly conserved among widely divergent organisms (68).

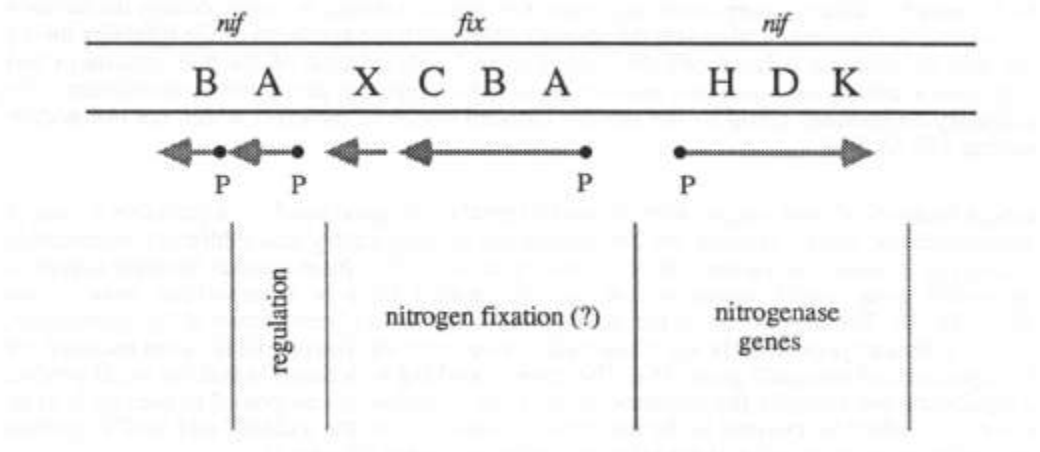


Figure 6.. Organization of nitrogenase (nil) and nitrogen fixation (fix) genes of Rhizobium trifolii.

The location of non-nitrogen-fixing (Fix-) sym plasmid mutants in R. leguminosarum and R. trifolii have been shown to occur specifically in two regions, one within 4kb of the nitrogenase genes and the other about 20 to 30kb from this region. At present, a cluster of genes involved in conferring a Fix+ phenotype in R. meliloti occupies about 14 to 15kb of which 12 to 13kb is indispensable for the Fix+ phenotype. Extensive Tn5-directed mutagenesis of this region has demonstrated the presence of several independent transcriptional units which are involved in the symbiotic phenotype (69). In R. trifolii the *nif*HDK gene cluster has associated with it the fixABCX genes, the *nif*B gene and the regulatory *nif*A gene (Figure 6). The precise role of these fix genes is still unknown although preliminary evidence suggests that one of them may encode a ferridoxin electron-transport function (J.M.Watson, pers. comm.).

Regulation of transcription from the promoters of these *nif* and fix genes (Figure 6) is known to be dependant upon the presence of the regulatory gene *nif*A. The Rhizobium bacteria in the nodule utilize an as yet unknown mechanism to induce first the *nif*A regulatory gene and

consequently the Fix+ phenotype. It is not known if this trigger occurs directly in response to a specific plant signal or if it occurs indirectly as a result of the physiological environment which exists in the nodule. Recent proposals by Kahn et al .(70) present an attractive hypothesis in which low levels of certain amino acids used as carriers of the carbon skeletons used for Rhizobium energy metabolism trigger nitrogen fixation in order to replenish amino acid carrier pools.

Future applications of rhizobium bacteria to agriculture

Inoculation of legumes with specific rhizobia has been widely used this century as an insurance policy to ensure that nitrogen fixation takes place and hence reduce the need for nitrogenous fertilizers. Undoubtedly this will continue to be of vital importance to agriculture. The less obvious but perhaps more wide spread use of Rhizobium bacteria to future agricultural practice is one of "engineering" the rhizosphere of crop plants, with the central aim to reduce production costs (8,21). Rhizobia can be used as vehicles (vectors) to deliver specific compounds to the soil-plant environment. These substances range from pesticides to herbicides and are aimed to lower the "disease load" on plant roots and provide promotion of plant growth (e.g., Table 2) .

A wealth of physiological, biochemical and molecular biological data is now available about Rhizobium bacteria. In particular, there is detailed information available on three *nod* gene promoters which are inducible by flavones released from plant roots. One of these promoters has recently been fused to an insecticide gene and the construction (when expressed in Rhizobium cells) results in insecticide release from the bacteria (Agrigenetics Research Corporation). There still are however two essential problems to the use of such constructions for biological control. The first involves the permission from environmental protection agencies to release "engineered" organisms into the environment. However, because Rhizobium bacteria have been used for such a long time as a soil inoculant in many different countries and on various soil types without any obvious defective side effects, it is likely that such constructions made in rhizobia will get the first permission to progress with extensive field testing (71). The second problem involves our understanding of microbial ecology. We are developing a sophisticated knowledge about the genetic organization of the Rhizobium cell. However, our ability to genetically manipulate Rhizobium bacteria into valuable commercial strains by a direct approach is still inhibited due to our lack of understanding of the genetic basis of strain competition and the performance of strains within the microbial ecology of the soil.

Table 2. Future possible contributions of Rhizobium to agriculture.

1. Cloned *Rhizobium* genes as probes for bacterial strain identification. Development of an understanding of microbial ecology in soil and plant rhizospheres.

2. Construction of novel *Rhizobium* strains by genetic manipulation
   - more competitive strains
   - acid tolerant strains
   - strains more tolerant to various metals (etc)

3. *Rhizobium* as a vector for delivery of specific compounds
   - fungicides
   - nematocides
   - bacteriocides

4. Extension of *Rhizobium* host-range
   - to other legumes
   - to tree legumes
   - to some non-legumes (?)

5. Using specific *Rhizobium* mutants as "probes" of plant biological responses
   - identification of plant resistance genes
   - identification of plant genes involved in nitrogen fixation

It has been found that a major constraint on the successful use of new Rhizobium inoculant strains is the competition between introduced and indigenous strains of rhizobia in the soil. Native rhizobia can often exhibit poor symbiotic efficiency and yet form the majority of nodules on host plants even if the legume seeds or the soil are inoculated with more desirable commercial Rhizobium strains (72,73). Genetic improvement of Rhizobium bacteria for greater nitrogen-fixing capacity or for specific rhizosphere functions will be of limited value unless a greater understanding is obtained concerning the ability to control strain competition in soils. There is a real need therefore, to come to terms with this complex problem of strain competition and the even more general problem of what are the selection pressures operating in the soil and root rhizosphere environments.

Microbial ecology in soil and plant rhizospheres

At present, there are readily available cloned genes and specific fragments of Rhizobium DNA which could be used as molecular "probes" for the precise identification of a particular inoculant strain in soil or in legume root nodules. Screening of soil/nodule inhabitants could test for, (a) the presence of chromosomal and plasmid genes characteristic of a particular species; (b) specific Sym plasmid genes (such as, repeated DNA sequences, *nod*-box sequences, *nod*, *nif* and fix genes); (c) strains with characteristic indigenous plasmid profiles.

The careful use of specific DNA probes could enable a precise bacterial species and strain identification to be made and thus provide the foundation of a "molecular taxonomy" of the presence of different bacteria in a particular region. A precise identification of which bacteria occupy a particular niche also will enable an analysis of whether there is a transfer of genetic material in the soil environment (that is, is there a "gene flow" between the different bacterial members of a particular niche).Some evidence from a recent set of experiments (74), suggest that transmission of a Sym plasmid can occur between Rhizobium strains in a soil environment. The results of these type of studies are crucial to strain construction programs and possibly the permission to use Rhizobium as a vector for the delivery of specific compounds to the rhizosphere of different crop plants.

Construction of novel rh1zobium strains by genetic manipulation

a) More competitive rhizobium strains

Recent observations of ours have opened an avenue of approach to creating more competitive commercial inoculum strains of Rhizobium. Firstly, a series of findings have shown that different Rhizobium strains vary in their ability to rapidly distort and invade root hairs. Moreover, a correlation existed between nodule occupation (i.e. successful invasion) and the rapidity of early invasion. Strains which rapidly induced nodulation genes were more competitive in laboratory experiments.

Secondly, studies with subterranean clover plants possessing two equally-infectable and robust lateral root systems ("split roots") have been used with several specific mutant strains (derived from R. trifolii strain ANU843) to investigate the systemic plant response induced by infective Rhizobium strains. This plant response controls subsequent nodulation on the plant. When strain ANU843 was inoculated onto both parts of the root system simultaneously or 24h, 48, 72 or 96h apart, an inhibitory response occurred which retarded nodulation on the part of the root exposed to the delayed inoculum. Inhibition only occurred when the delay period between inocula on each root was greater than 24h (75). Furthermore, the plant inhibitory response was initiated only by infective strains of Rhizobium, as pre-exposure of one root system to non-nodulating strains did not retard the ability of the wild type strain to nodulate the opposing root (even when the delay period was 96h). Moreover, the use of specific, Tn5-induced mutants subtly impaired in their ability to nodulate, demonstrated that the plant could effectively and rapidly discriminate between infection initiated by the parental or by the mutant strains (75). When inoculated alone onto clover plants, these mutant strains were able to infect the most susceptible plant cells at the time of inoculation and induce nitrogen-fixing nodules. However, separate but simultaneous inoculation on different parts of the root system of the parent and the mutant strains resulted in the complete inhibition of nodulation of the mutant strains. We concluded that the mutants were affected in their "competitive ability" and this was reflected by poor nodule occupancy when co-inoculated with the parent strain onto a single root system. In addition, using this plant assay technique; we have shown that the competitiveness of a strain can be altered by the alteration of the expression of the *nod* genes. Pre-exposure of some poorly-competitive strains to the plant signal 7,4'-dihydroxyflavone (DHF) for 4 hours, allowed them to out-compete normally more successful strains (Sargent et al. in prep).

Thus, the split-root system forms the basis of a simple screening system for the ranking of competitiveness of various rhizobia on small seeded legumes and the characterization of mutant rhizobia which are less competitive. Part of the genetic ingredients of a future inoculum strain will be the rapid induction of its nodulation genes by the available plant signals under various soil conditions. It will still take a number of years to acquire the necessary information on the Rhizobium genes which influence strain competitiveness and favoured occupancy of the rhizosphere and root surface. But such information is fundamental if some of the other proposed uses of Rhizobium bacteria are to be achieved.

b) Construction of acid tolerant rhizobium strains

Extensive use of subterranean clover-based pastures throughout Southern-Australia has caused a significant decline in soil pH (76,77). Presently as much as 14 million hectares of agricultural soils throughout southern Australia are reported to be affected by increasing soil acidity (78). Although the rate of acidification of soils varies considerable depending on soil type and environmental factors, it has been estimated that the time required for a decrease of one pH unit ranges from between 30 and 100 years (76). Considering that currently more than 75% of improved pastures throughout southern-Australia are less than 30 years old, it may be anticipated that the area of agricultural soils affected by low pH will increase dramatically in the future.

In an acid soil, low pH (hydrogen ion toxicity) and/or numerous nutrient imbalances (e.g., calcium, molybdenum deficiencies, and aluminium, manganese toxicities) can affect the survival of Rhizobium and/or the establishment of an effective symbiosis (79,80,81,82,83). Subterranean clover plants themselves are moderately tolerant to acidic soils, however, R. trifolii and consequently nodulation have been demonstrated to be sensitive to low pH and associated acid soil factors (84,85). In culture studies,

the growth rate of R. trifolii is reduced at pH less than 5.0. At these pH's rhizobia have an extended "lag phase" and slower "exponential" phase of growth (81, Richardson unpublished). Because R. trifolii cells in early exponential growth are those which are able to rapidly derepress their *nod* genes in the presence of DHF (36), it is feasible that low pH could directly affect *nod* gene expression simply due to its affect on growth of the cells. Indeed, it has recently been shown that *nod* gene expression does in fact occur at lower pH's in acid tolerant Rhizobium and that expression was entirely related to the ability of the bacterium to grow at the low pH (Richardson et a!., in prep).

Considering that root hairs are "transiently infectable" (51) for short periods of time (as short as 6 hours) failure to initiate nodules under acidic conditions is most probably due to the inability of acid sensitive strains (which includes currently used inoculant strains, Richardson unpublished) to rapidly express nodulation genes and initiate invasion of the "infectable" root hairs. We therefore believe that the selection of rhizobia capable of tolerating low soil pH and/or high levels of aluminium could be potentially useful. Using Tn5 mutagenesis we have identified loci on the chromosome and on a non-Sym plasmid which are involved in acid tolerance of R. trifolii bacteria in laboratory media (Richardson and Rolfe, unpublished). Molecular cloning of these regions should enable the transfer of "acid tolerance" to desirable inoculant and/or competitive strains. Moreover, by using drug resistant plasmids containing large insertions of chromosomal DNA (R-prime plasmids)(86), it has been possible to construct strains with different levels of tolerance to environmental factors such as heavy metals. These same techniques are being used to construct different R-prime plasmids containing (a) Rhizobium genes that are associated with the acid tolerance phenotype and (b) R-primes containing genes important to Rhizobium strain competitiveness (Table 2).

Rhizobium as a delivery vehicle

Because rhizobia can successfully inhabit the rhizosphere of both leguminous species and grasses (8,87), they have potential use as vectors for biological control. As part of an overall strategy to reduce the cost of farming and the dependance on the use of insecticides and herbicides, specific inoculant bacteria could be used to introduce specific compounds to the rhizosphere of particular crop plants. This would be part of a policy of biological control of pests and would involve using appropriate Rhizobium strains as a vehicle for the secretion of specific compounds against, for instance, fungi, nematodes, bacteria or insects which cohabit in the rhizosphere and which may be detrimental to plant growth. These secreted substances could either act within the rhizosphere of a legume or non-legume or be released in nodules to be transported systemically throughout the nodulated plant.

Extension of rhizobium nodulation to other plants

The accumulation of basic information on the structure, function and regulation of bacterial genes involved in the initiation and development of symbiotic biological nitrogen fixation should enable the development of Rhizobium strains with broader host-range properties. Certain Rhizobium strains can infect the non-legume Parasponia (88,89) but they do not infect the closely related plant Trema cannabina (90). A careful analysis of the association of Rhizobium bacteria with the roots of these two plant species should help in the evaluating the feasibility of extending the Rhizobium symbiotic system to legumes which are not nodulated, to tree legumes and possibly to other non-legumes. At the present time, it seems that the nodulation of the monocotyledonous plants (such as wheat), has a low probability. However, fundamental information concerning he colonization and saprophytic properties of Rhizobium strains may offer clues to help the re-examination of bacterial associations with roots of non-legumes.

Rhizobium strains as probes of plant responses

The inducible "hypersensitive reaction" (HR) is a major component of the disease resistance mechanism in plants. Induction of the HR system by an infecting microbe causes host cell death and a series of accompanying events including changes in oxidative metabolism, accumulation of toxic compounds and often the lignification of plant cell walls (91). Nodulation by rhizobia is only possible because the hypersensitive reaction is either bypassed or not induced by the invading Rhizobium bacteria. We consider that particular mutants of Rhizobium can be used as "probes" to understand the complex

interaction of the plant defence mechanisms against "irritant" microorganisms. Although rhizobia are classified as a subgroup of the Rhizobiaceae (which includes the well-known plant pathogen Agrobacterium), they are infrequently considered as plant-pathogenic or parasitic bacteria. By viewing the Rhizobiumlegume interaction from a phytopathological standpoint, we consider an investigation can be made of those components of the bacterial-plant interaction which have general significance for plant biology (7). Such information could be used in plant breeding programs to help in the analysis of the segregation of resistance genes.

The nature and number of plant genes which are essential for symbiotic nitrogen-fixation has just begun to be addressed. In the establishment of a nitrogen-fixing root nodule, complex interactions involving the expression of both plant and bacterial genes occur. Intricate structural and biochemical changes which involve each partner result in the assimilation of nitrogen by the host plant. A group of plant gene products, termed nodulins, have been identified and found to occur specifically in the nodules (92,93,94,95). During root nodule development at least 20 nodule specific genes are expressed. At the present time however, many of these proteins have remained uncharacterized.

In view of the specific interaction between Rhizobium and its host plant, it appears likely that the expression of nodulin genes is controlled in some way by the presence of the bacteria. It seems likely that the first signal that activates expression of a nodulin gene(s) will be derived from the infecting rhizobia. Whether further signals that lead to expression of nodulin genes are derived from plant-encoded genes or also from the rhizobia is a matter of speculation at this stage. Future use of specific mutants of Rhizobium will advance our understanding of the mechanisms involved in both plant pathology and normal nitrogen-fixing symbiotic associations. This information and the genetic probes that will be generated in these studies can then be used in diagnostic kits to follow gene segregation in plant breeding programs and hence accelerate the rate of developing new plant varieties.

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