The Development of *Rhizobium* Genetics

The Fourth Fleming Lecture

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INTRODUCTION

The rhizobia are Gram-negative bacteria of major agricultural importance because of their ability to form nitrogen-fixing nodules on the roots of leguminous plants. The nitrogen fixation genes are carried by the rhizobia and are possibly only expressed in nature within the root nodule. Usually a given strain of *Rhizobium* can nodulate only a limited number of legume species. For temperate species this has given rise to a taxonomic classification based on the range of host plants that are nodulated. For example, *Rhizobium trifolii* includes those rhizobia which (ideally) nodulate only clovers (*Trifolium* spp.) which, in turn, are (ideally) nodulated only by strains of *R. trifolii*. However, while this grouping works reasonably well with temperate legumes, the far more numerous tropical legumes and rhizobia appear not to show the same host-*Rhizobium* specificity. A more useful characterization is on the basis of growth rate which reflects basic metabolic differences: temperate rhizobia on the whole are fast growers (taking 3 to 7 d to form colonies on agar plates) while the others are generally slow growers (7 to 21 d).

Root nodules are formed as the result of a series of poorly understood interactions between the appropriate *Rhizobium* species and its host plant. There is evidence that the population of rhizobia in the soil is stimulated by legumes (Robinson, 1967) but insufficient data are available to determine whether this is specific for a given host-*Rhizobium* combination, or is a general response of rhizobia to the presence of leguminous plants. Recognition, which may involve lectin binding (Dazzo & Hubbell, 1975), probably occurs before entry into the root, usually through root hairs or, more rarely, through breaks in the root occurring, for example, at the emergence of lateral roots. Within root hairs the bacteria are always enclosed within a tube, the infection thread, which is surrounded by a pectic and cellulose wall material that is continuous with the plant cell wall. The infection thread branches and penetrates through cells within the cortex. This growth is correlated with (stimulates?) cell division in the inner root cortex and the growth of the root nodule. Nodule formation, unlike tumour formation by *Agrobacterium tumefaciens* (another member of the *Rhizobiaceae*), is controlled, and a differentiated structure is formed.

Within the nodule the rhizobia differentiate into bacteroids, which are the nitrogen-fixing forms. The plant produces globin (Sidloi-Lumbroso *et al.*, 1978); this combines with haem produced by the rhizobia (Cutting & Schulman, 1969) to form leghaemoglobin, which appears to be a prerequisite for a nitrogen-fixing legume nodule. Once infection has occurred the plant provides the rhizobia with all their nutrient requirements and, in turn, receives fixed nitrogen when a nitrogen-fixing strain of *Rhizobium* is involved.

We know that rhizobia can produce indole-acetic acid (Dullaart, 1970) and very small amounts of cytokinins (Phillips & Torrey, 1972), both of which are important plant hormones. There is also an indication that rhizobia can produce small amounts of polygalacturonases which may be important in obtaining an entry into the root and in the growth of

the infection thread within the root. The significance and role of any of these substances during the differentiation remains to be established. How many other stimulatory substances are produced (or need to be produced) by either partner is unknown.

EARLY GENETIC STUDIES (1941-1970)

Beijerinck in 1888 first reported the isolation of bacteria from legume root nodules and demonstrated that the purified bacteria were able to form nodules on plants grown from sterilized seed (see Stanier *et al.*, 1968). Studies of gene transfer can be said to have started in 1941, when Krasilnikov (1941, 1945) studied the effect of growing rhizobia in culture filtrates from different *Rhizobium* species and reported that the ability to nodulate clover and lucerne was transferred between species. This work cannot be readily assessed; it may have been the first report of transformation in *Rhizobium*. Since that time there have been numerous publications on transformation in *Rhizobium*, the majority being on the transfer of drug resistance. In very few cases have these reports presented good linkage data and, as a result, the value of transformation for the genetic analysis of *Rhizobium* has been limited.

Transduction, like transformation, involves the transfer of small segments of DNA, which limits its value for primary linkage studies. Surprisingly, despite many reports of the isolation of phages for *Rhizobium*, by 1970 the only reports of transduction were by a single author, Kowalski (1967). This probably reflected a lack of interest in transduction since Kowalski (1970) has reported that many isolates of *R. meliloti* phages are able to mediate generalized transduction and recently Buchanan-Wollaston (1979; personal communication) has shown that several *R. leguminosarum* phages are able to transduce this species and one was also shown to transduce DNA between *R. leguminosarum* and *R. trifolii* and within strains of *R. trifolii*.

Conjugation, which involves the transfer of large pieces of DNA, should be the most suitable method of gene transfer for establishing linkage maps in micro-organisms. A linkage map for *R. lupini* was reported by Heumann (1968). His work involves one of the only bacterial conjugation systems known where pair formation and gene transfer are apparently not dependent on conjugative plasmids. Two chromosomally located sex factors (Fc and Fd) independently act as origins of DNA transfer (Heumann *et al.*, 1971), in a similar manner to Hfr strains of *Escherichia coli*, though without a known extra-chromosomal state, such as F⁺. Unfortunately all strains involved in the mapping work were derived from an original strain that was non-nodulating. Thus it has not been possible to utilize this system for the genetic analysis of symbiotically important genes.

RECENT GENETIC STUDIES (1971-1979)

By 1970, it was obvious that fast-growing rhizobia such as *R. leguminosarum*, *R. trifolii*, *R. meliloti* and *R. phaseoli* were amenable to genetic manipulation. Mutation procedures had been described (Heumann, 1968; Scherrer & Dénarié, 1971) and a wide range of auxotrophic and drug-resistant mutants had been produced. Many papers on transduction, and particularly transformation, had been published. However, the establishment of linkage maps in nodulating strains of *Rhizobium* seemed a remote possibility. A conjugation system utilizing a sex factor with multiple sites of interaction with the chromosome was an obvious requirement for mapping studies. Because conjugation had not been reported for the species mentioned above, there were no known sex factors and there was no basal level of recombinant formation to attempt to improve upon. This is a particularly frustrating aspect of starting genetic studies with 'new' species, since any change in the way crosses are performed cannot be compared with previous methods to determine whether they are beneficial or detrimental. We now know that mutations conferring streptomycin and rifampicin resistance are closely linked in fast-growing *Rhizobium* species. These easily

selected markers thus provide a convenient method for demonstrating gene transfer; we have used this linkage in a quick screen to demonstrate recombination between different *Rhizobium* species (Johnston & Beringer, 1977) and to screen *Rhizobium* species for intrinsic sex factors.

The transfer of indigenous *Rhizobium* plasmids between rhizobia has been reported by Higashi (1967), Cole & Elkan (1973) and ourselves (Johnston *et al.*, 1978*c*; Hirsch, 1979). However, except for our recent studies with *R. leguminosarum* plasmids (Hirsch, 1979), linkage mapping by conjugation has depended on the P1 group R plasmids RP4 and R68.45 which confer resistance to the antibiotics carbenicillin, tetracycline and kanamycin/neomycin. The P1 group R plasmids were originally found in *Pseudomonas aeruginosa* and shown by Datta *et al.* (1971) to be transmissible between a wide range of Gram-negative bacteria. Their ease of selection in crosses, relatively high frequency of transfer (10^{-1} to 10^{-3}) and stability in *Rhizobium* made them obvious candidates for developing linkage mapping in *Rhizobium*.

Initial studies with RP4 in *R. leguminosarum* (Beringer, 1974) showed that conjugation could occur, both between rhizobia and between *R. leguminosarum* and *E. coli*. The frequencies of transfer in the various cases were similar; about 10^{-2} per recipient. However, RP4 was a poor sex factor in the *R. leguminosarum* strains used and too few recombinants were formed for it to be useful for mapping purposes. Two approaches were taken to obtain derivatives of RP4 with improved chromosome donor properties; neither was successful. The first approach was to construct R-primes by inserting *Rhizobium* DNA fragments prepared by *EcoRI* digestion into the *EcoRI* site in RP4. F-prime mobilization of *E. coli* chromosomal genes is 10^2 to 10^7 times more efficient than that by F^+ donors and we hoped that this might also have been the case with R-prime mobilization. Two R-primes carrying *R. leguminosarum* DNA were made and tested but no increase in recombinant formation for any marker was detected (Jacob *et al.*, 1976). We have since looked for mobilization of DNA from the region of the *phe-I* mutation in our *R. leguminosarum* strain carrying an R68.44-*phe-I*⁺ plasmid. No increase in chromosome mobilization was detected (J. E. Beringer, unpublished results). Recently, Julliot & Boistard (1979) have shown that four of 16 R-primes constructed *in vitro* using *R. meliloti* DNA promoted high-frequency polarized chromosome transfer. It is conceivable that the non-functional R-primes did not carry chromosomal DNA, though this does not explain our results with the *phe-I*⁺ prime.

Our other approach to obtain plasmids with better chromosome donor properties was to test recombinants as donors in further crosses. This screen was based on the idea that since the recombinants had arisen at very low frequency they might have been due to rare plasmid modifications in the donors. More than 200 recombinants were tested, representing different initially selected markers. No high-frequency generalized chromosomal donors were obtained, though two R-primes were isolated. While we were carrying out these experiments Haas & Holloway (1976) were doing similar experiments with *Pseudomonas aeruginosa* using R68 as the sex plasmid. They were able to show that a high proportion of recombinants, especially those initially selected for *argB*⁺, carried plasmids with greatly enhanced chromosome donor properties. R68.44 and a derivative, R68.45, were two of these high-frequency chromosome donor plasmids. The generosity of Haas and Holloway in distributing strains carrying these plasmids so soon after isolating them has been rewarded by the success that so many laboratories have had in developing linkage maps in other species and genera of bacteria. Within a year of receiving R68.45 we had satisfied ourselves that *R. leguminosarum* had a single circular chromosome.

The rapid rate with which linkage maps were constructed in *R. meliloti* (Kondorosi *et al.*, 1977) and *R. leguminosarum* (Beringer & Hopwood, 1976; Beringer *et al.*, 1978*a*) by use of R68.45 and in *R. meliloti* by use of RP4 (Meade & Signer, 1977) was mainly due to the relatively large size of transferred DNA fragments and the apparently very large number of sites of interaction of these plasmids with the chromosome. Kondorosi *et al.* (1977) have

calculated that the average size of DNA transferred during R68.45-promoted conjugation in *R. meliloti* is about one-third of the chromosome. When such large fragments of DNA are transferred, linkage between fairly distant markers is relatively easy to demonstrate and thus large numbers of genetically marked strains are not needed to construct linkage maps. It is surprising that while R68.45 was isolated in *P. aeruginosa* (strain PAO1) and transfers markers from most parts of the chromosome in that strain it has not been possible to demonstrate the circularity of its chromosome (Holloway *et al.*, 1979), though R68.45 has been used to show circularity in strain PAT (Holloway *et al.*, 1979). Another unsolved question with these R plasmids is the reason why RP4 and R68 are so poor at mobilizing chromosomal genes in *E. coli* and *R. leguminosarum*, while RP4 is so efficient in *R. meliloti*.

An interesting sideline arising from mapping studies with the R plasmids has been the isolation and intergeneric transfer of R-primes constructed *in vivo*. Most 'recombinants' formed in crosses between *R. meliloti* as donor and *R. leguminosarum* as recipient are R-primes (Johnston *et al.*, 1978*b*). These presumably constitute the only 'recombinants' because homology between the *R. meliloti* and *R. leguminosarum* chromosomes is so poor that *R. leguminosarum* is analogous to a *recA* recipient in these crosses. These R-primes are transmissible to *P. aeruginosa* and *E. coli* and allow us to study the function of *Rhizobium* genes in these species. Expression of *R. meliloti* *trp*⁺ alleles in *trp* mutants of *P. aeruginosa* was detected by growth of tryptophan-independent colonies. However, none of the six *R. meliloti* *trp*⁺ genes was able to suppress the Trp⁻ phenotype of *E. coli* *trp* auxotrophs (Johnston *et al.*, 1978*a*). By plating *E. coli* carrying one of the *trp*⁺ primes on media without tryptophan, rare Trp⁺ colonies arose which were shown to carry altered R-primes. The basis for the alteration of the R-primes is unknown.

Before discussing indigenous plasmids in *Rhizobium* it is relevant to mention mutagenesis in *Rhizobium* and describe some of the problems associated with the handling of symbiotic mutants. Maier & Brill (1976) and ourselves (Beringer *et al.*, 1977) have shown that mutants of *Rhizobium* that are unable to carry out a normal symbiosis can be isolated after 'conventional' chemical mutagenesis. Classically such mutants are classified into two phenotypes; non-infective (Inf⁻) and ineffective (Eff⁻). Inf⁻ are those mutants which do not infect the legume host to form nodules, while Eff⁻ mutants form nodules which do not fix nitrogen. Because it is difficult to demonstrate convincingly that a strain is unable to infect a legume root, many strains are called Inf⁻ on the basis that nodules are not seen. Therefore we have introduced the phenotypic designations Nod and Fix to clarify the description of mutant phenotypes (Brewin *et al.*, 1980). Nod⁻ mutants are those that do not produce a visible nodule and Fix⁻ those that produce nodules which do not fix nitrogen. Operationally, Nod replaces Inf and Fix replaces Eff.

By definition, Nod⁻ and Fix⁻ mutants have a phenotype that can be expressed only after adding the bacteria to the appropriate host plant. Thus the isolation and mapping of these mutants requires large numbers of plant tests. To circumvent this problem we wished to make symbiotically defective mutants by transposon mutagenesis using drug-resistance transposons, since once a mutant was identified it would have two phenotypic properties: the symbiotic defect and the drug resistance. Because the mutation is caused by inactivation of the symbiotically important gene by the transposon, mapping of that gene can be done without recourse to plant tests. The segregation of the gene in crosses can be followed by studying the segregation of the drug resistance, which can be tested on Petri dishes. Van Vliet *et al.* (1978) showed that when bacteriophage Mu was inserted into the P1 group plasmid RP4 this plasmid was no longer able to be inherited by *Agrobacterium tumefaciens* in crosses with R⁺ *E. coli* donors. Thus, when a transposon was present in the R plasmid it could be introduced into *A. tumefaciens* on a vector which was not replicated, selection for inheritance of the transposon effectively selecting for transposition into the chromosome or plasmids of the recipient. This technique also works well for *Rhizobium* (Beringer *et al.*, 1978*b*).

We have used the transposon Tn5, which confers kanamycin resistance, for these studies and have shown that it can integrate into many sites within the chromosomes of *R. leguminosarum*, *R. trifolii* and *R. phaseoli* (Beringer *et al.*, 1978*b*) and also into indigenous plasmids within *R. leguminosarum* (Johnston *et al.*, 1978*c*). Hirsch (1979) had previously shown that indigenous plasmids carried bacteriocin production genes and was able to follow their transfer by screening for bacteriocin production by recipients. Insertion of Tn5 into these plasmids meant that they could be selected directly, as though they were classical R plasmids. One of these bacteriocinogenic plasmids was shown to transfer the ability to nodulate peas when transferred from *R. leguminosarum* to *R. trifolii*, *R. phaseoli* and a strain of *Rhizobium* able to nodulate *Cicer* (Johnston *et al.*, 1978*c*). Transfer of the Tn5-coded drug resistance to *R. meliloti* and *Agrobacterium tumefaciens* was obtained, but these strains were unable to nodulate peas (A. W. B. Johnston, unpublished results). These results are consistent with the known taxonomic relationship of these species, *R. leguminosarum*, *R. trifolii* and *R. phaseoli* being more closely related to each other than to *R. meliloti* (Graham, 1964). They are also consistent with data obtained from crosses between these species. In crosses between *R. meliloti* and *R. leguminosarum* there appears to be little chromosomal homology and most recombinants are R-primes (Johnston *et al.*, 1978*b*). However, the frequency of recombinant formation and linkage between markers in crosses of *R. leguminosarum* with *R. trifolii* or *R. phaseoli* is about the same as between derivatives of the same isolate of *R. leguminosarum* (Johnston & Beringer, 1977). These crosses have also shown that genes associated with host range are probably plasmid-borne in these three species, since the replacement of *R. leguminosarum* chromosomal DNA with that from the other species did not alter the host range of any recipient tested (Johnston *et al.*, 1978*c*; J. L. Beynon, unpublished observations). Thus it appears that these 'species' are chromosomally one species which is able to nodulate and fix nitrogen in roots of a particular group of legumes if the appropriate plasmid is present.

It appears that future genetic studies of *Rhizobium* will initially be dominated by studies of plasmids. Higashi (1967) was the first to suggest that host-range genes might be plasmid-borne in *Rhizobium* and since that time many workers have shown that *Rhizobium* strains carry plasmids (Sutton, 1974; Nuti *et al.*, 1977; Casse *et al.*, 1979). Dunican and co-workers have reported that nitrogen-fixation (Nif) genes are likely to be plasmid-borne and are transmissible to *Klebsiella* (Dunican *et al.*, 1976; Stanley & Dunican, 1979). The transfer of *Rhizobium* Nif genes to *Azotobacter* (Page, 1978) and the finding that *Rhizobium* plasmid DNA hybridizes with *Klebsiella pneumoniae* Nif DNA (F. Cannon, personal communication) suggest that nitrogen-fixation as well as host-range genes may be plasmid-borne. We now have good evidence from studies with *R. leguminosarum* that genes required for nodulation (Nod) and the establishment of a nitrogen-fixing nodule (Fix) are plasmid-borne in at least two strains of *R. leguminosarum*.

CONCLUSION

The purpose of genetic studies has primarily been to facilitate our attempts to understand the *Rhizobium*-legume symbiosis. To this end we would like to determine the number, function, regulation and genetic arrangement of genes involved in the symbiosis for both *Rhizobium* and the legume host. *Rhizobium* genetic studies have reached a point where there is no obvious limitation in making mutants and manipulating genes in fast-growing *Rhizobium* species. The range of symbiotically defective mutants of *Rhizobium* which are presently available is limited by the difficulties in handling the very large plasmids that appear to carry some of these genes and by the limit on the rate of analysing symbiotically defective mutants imposed by plant tests.

The slow-growing rhizobia, such as *R. japonicum* which nodulates soybeans, have proved to be very difficult to handle genetically. It is still hard to obtain auxotrophic mutants, and no linkage maps have yet been published. This is unfortunate because of the obvious

economic importance of the soybean (the host for *R. japonicum*) as a grain legume and because some of the slow-growing rhizobia are still the only rhizobia which can routinely be shown to fix significant amounts of nitrogen as free-living bacteria. However, this is unlikely to impede our understanding of the symbiosis because the fast-growing rhizobia are intrinsically easier to handle as bacteria. Indeed, the range of genetic techniques now available to analyse them is such that our understanding of the symbiosis will undoubtedly progress rapidly over the next few years.

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