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INVITED REVIEW

THE GENETIC ANALYSIS OF *RHIZOBIUM* IN RELATION TO SYMBIOTIC NITROGEN FIXATION

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SUMMARY

In the last few years there has been an increasing interest in the genetics and molecular biology of *Rhizobium*, the bacterium that is responsible for inducing nitrogen-fixing nodules on the roots of leguminous plants, and in

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this paper we have reviewed recent work in this field. We have considered, in turn, the development of systems of chromosomal gene transfer and mapping in *Rhizobium*, the recent studies implicating indigenous plasmids as important determinants of symbiotic functions and, finally, the use of *Rhizobium* mutants as aids in the biochemical analysis of the symbiosis.

1. Introduction

To an increasing degree the genetic knowledge and precedents found in the intense genetic study of *Escherichia coli*, its viruses, and its plasmids, have been reapplied on a more modest scale to facilitate the analysis of other Gram negative bacterial species. Such species have been chosen on the basis of their economic, medical or ecologial importance or on the very fact that they are strikingly different from *E. coli* in terms of taxonomic, biochemical or morphological criteria (see review by Levinthal, 1974).

There is no doubt about the economic importance of the genus *Rhizobium*, whose members are responsible for the induction of nitrogen-fixing nodules on the roots of leguminous plants. As a result of this symbiosis such crops as soya, *Phaseolus* beans, peas, lentils, chick peas, clover, alfalfa, and others may be grown with no requirement for nitrogenous fertilizer. On a global level, *Rhizobium* in legume root nodules may each year reduce as much as 20×10^6 tonnes of atmospheric nitrogen to ammonia.

There have been several reviews describing the morphological and biochemical changes that occur during the infection process and the development of the mature nodule (Dart, 1975; Newcomb, 1976; Beringer *et al.*, 1979). Briefly, the bacteria usually penetrate the plant *via* a root hair cell and are accommodated in an invagination of the cell wall (the infection thread) which grows through the cortical cells. By unknown mechanisms the presence of *Rhizobium* in the root induces plant cell proliferation. The precise morphology and development of nodules vary between different legumes, but the nodule is an organized structure with a well established vascular system. As the nodule develops, the bacteria are released into the cytoplasm from the infection threads, and are surrounded by plant membranes. During this time the released bacteria differentiate into bacteroids, the form that is responsible for the fixation of nitrogen. Typically, these bacteroids are pleiomorphic, have lost much of their cell wall, and are less electron dense than the free-living bacteria. The ammonia that is produced by the bacteroids passes to the surrounding plant cytoplasm where it is assimilated into amino acids.

One aspect of the symbiosis is its specificity whereby particular legume species are nodulated only by certain *Rhizobium* species; indeed *Rhizobium* species are defined on the basis of their host range. In this paper a number of *Rhizobium* species will be mentioned and the genetic basis of host range will be discussed in some detail. These *Rhizobium* species are shown in table 1 together with the names of some of their better known host plants. This classification, although a useful shorthand, leaves much to be desired (Wilson, 1944). For example, on the basis of numerical taxonomy, strains of *R. leguminosarum*, *R. trifolii* and *R. phaseoli* show as much intra- as inter-specific variation (Graham, 1964*a*) and indeed it appears that the host range of strains classified in these three species depends on their complement of plasmids (see below).

TABLE 1

Host range of some species of Rhizobium

Rhizobium species	Host legume nodulated
R. leguminosarum	Pea (*Pisum spp*), *Lathyrus*, *Lens* and *Vicia spp*
R. trifolii	Clover (*Trifolium spp*)
R. phaseoli	*Phaseolus* beans
R. lupini	Lupin (*Lupinus spp*)
R. meliloti	Alfalfa (*Medicago spp*)
R. japonicum	Soya bean (*Glycine spp*)

Although the history of *Rhizobium* genetics may be traced back to 1941 (Krasilnikov, 1941*a*) it is only in the last few years that such studies have developed sufficiently to be able to form a framework for the analysis of the *Rhizobium* genes that determine symbiotic functions. Such studies may be grouped for the purposes of this review into three main areas:

(1) Development of procedures for gene transfer, leading to formal genetic analysis of the *Rhizobium* chromosome.
(2) Genetic and physical characterization of plasmids with particular reference to their role in symbiosis.
(3) The use of induced and naturally occurring genetic variation to dissect the developmental changes that occur during infection and nodulation.

2. CHROMOSOMAL RECOMBINATION

(i) *Transformation*

Transformation may have been the basis of the first report of gene transfer in *Rhizobium* (Krasilnikov, 1941*a*). There have been many later reports of this method of genetic exchange; however, no transformation system has been developed to a stage suitable for fine scale chromosomal mapping. Perhaps more pertinent, in view of the role of *Rhizobium* plasmids in symbiosis, is the fact that transformation of *Rhizobium* with plasmid DNA has apparently not been reported. Clearly the development of transformation with plasmid DNA would be important both in the elucidation of the role of natural *Rhizobium* plasmids and in the development of recombinant DNA techniques. The fact that transformation with plasmid DNA occurs in *Agrobacterium tumefaciens* (Holsters *et al.*, 1978), another member of the Rhizobiaceae, offers hope for a similar system in *Rhizobium*.

(ii) *Conjugation*

The most detailed chromosomal mapping has arisen from studies of conjugal gene transfer. The first such reports (Heumann, 1968; Heumann *et al.*, 1971) involved a system of chromosome transfer in a strain of *R. lupini* which was apparently not mediated by any plasmid and in which there were two different regions of transfer. Unfortunately this strain failed to nodulate and hence these studies did not lead to the mapping of genes concerned with symbiotic functions.

More recent studies on conjugational chromosome transfer have made use of antibiotic resistance plasmids of the P1 incompatibility group, initially

identified in *Pseudomonas*. The most striking feature of such plasmids is their very wide host range among Gram-negative bacteria, including various *Rhizobium* species (Datta *et al.*, 1971; Beringer, 1974; Meade and Signer, 1977; Kondorosi *et al.*, 1977*a*; Kuykendall, 1979). In a strain of *R. leguminosarum* several P1 group plasmids were found to promote chromosomal recombination, but at frequencies too low (*c.* 10^{-9}) to allow chromosomal gene mapping (Beringer *et al.*, 1978*b*). Interestingly, one of these plasmids, RP4, promoted chromosomal recombination in a strain of *R. meliloti* at frequencies about 10^3-fold higher than in *R. leguminosarum* (Meade and Signer, 1977).

A successful attempt to improve on the usually low level of chromosome donor ability (Cda^+) of P1 group plasmids was made by Haas and Holloway (1976). They found that R68 mobilized the chromosome of *P. aeruginosa* strain PAO very inefficiently but when the rare recombinants were examined for Cda, some were up to 10^3-fold more efficient than R68; one of these, R68·45, was used in *P. aeruginosa* for mapping purposes. The molecular weight of R68·45 is larger than that of R68 by about $1{\cdot}5 \times 10^6$ and derivatives of R68·45 which have lost Cda^+ have also lost this extra DNA. It is tempting to suppose that the extra material is an insertion sequence whose presence is required for the increased Cda proficiency of R68·45 (Burkhardt *et al.*, 1979).

R68·45 can be transferred at high frequency from *P. aeruginosa* or *E. coli* to *R. meliloti* and *R. leguminosarum* where it mobilizes the chromosome efficiently (Beringer and Hopwood, 1976; Kondorosi *et al.*, 1977*a*; Beringer *et al.*, 1978*b*; Casadesus and Olivares, 1979*a*). This plasmid has been used to produce circular linkage maps in *R. leguminosarum* (Beringer *et al.*, (1978*b*) and two different strains of *R. meliloti* (Kondorosi *et al.*, 1977*a*; Casadesus and Olivares, 1979*a*). A circular chromosome in yet another *R. meliloti* strain was demonstrated by Meade and Signer (1977) who used the natural RP4 as a sex plasmid.

Mapping in these studies involved crosses between multiply auxotrophic or antibiotic resistant strains, with selection for the transfer of one allele. The selected recombinants were examined for the co-inheritance of the other, non-selected, alleles and the resulting co-inheritance frequencies were inversely related to map distance. Kondorosi *et al.* (1977*a*) converted the co-inheritance frequencies (c) into approximately additive map distances (d) by using a modification of the Wu equation as follows:

$$d = 1 - \sqrt[3]{c}.$$

Some features of the behaviour of R68·45-mediated recombination in *Rhizobium* were noted in all the above reports. These can be summarised as follows:

(a) Chromosome transfer is from the donor R^+ strain to the R^- recipient.
(b) All alleles, irrespective of map location, are transferred at approximately the same frequency to a particular strain.
(c) There is a large enrichment (10 to 100-fold) for the inheritance of the R plasmid among recombinant compared to non-recombinant recipients.
(d) Large regions of the chromosome (up to half) can be transferred and inherited.

(e) There is little multiple crossing-over; *i.e.*, if two outside markers are inherited, virtually all recombinants inherit any intervening non-selected donor alleles;
(f) The Cda^+ phenotype is unstable; up to 70 per cent of transconjugants have the low level mobilization frequency characteristic of the original R68 plasmid.

There is nothing immediately striking about any of the *Rhizobium* maps; for example there are no large silent regions. In each of the maps the genes for a particular biosynthetic function are more dispersed than in the Enterobacteriaceae, e.g., the genes for tryptophan biosynthesis, which comprise a single operon in *E. coli* (Bachmann *et al.*, 1976) and a single cluster in *Salmonella typhimurium* (Sanderson, 1970) are located in at least three distinct regions of the chromosomes of *R. meliloti* and *R. leguminosarum* (Kondorosi *et al.*, 1980).

The maps of *R. leguminosarum* strain 300 (Beringer *et al.*, 1978*b*) and those of two *R. meliloti* strains (Kondorosi *et al.*, 1977*a*; Meade and Signer, 1977) were compared by Kondorosi *et al.* (1980) using the mapping function described above. The map lengths of the three chromosomes were found to be very similar, as were the positions of many mutations for particular nutritional requirements, despite the fact that there is very little R68·45-mediated recombination between these two species (Johnston *et al.*, 1978c; Kondorosi *et al.*, 1980).

In contrast, R68·45-mediated crosses between strains of *R. leguminosarum*, *R. trifolii* and *R. phaseoli* yielded haploid recombinants for many alleles at frequencies similar to those found in crosses within derivatives of a single *R. leguminosarum* strain (Johnston and Beringer, 1977; J. L. Beynon, unpublished results) confirming the very close taxonomic relatedness of these three species (Graham, 1964*a*). The linkage relationships between various pairs of alleles were similar in the inter- and intra-specific crosses. One aim of these crosses was to map the genes responsible for determining host range. We have transferred, piece by piece, virtually the whole of the chromosomes of *R. trifolii* and *R. phaseoli* to *R. leguminosarum*, but there was no transfer of the ability to nodulate clover or *Phaseolus* beans. These negative results were circumstantial evidence that the genes determining host-range in these species might be extrachromosomal, a suspicion that was subsequently confirmed (see below).

As mentioned above, R68·45-mediated recombination between strains of *R. leguminosarum* and the more distantly related *R. meliloti* occurred at very low frequencies; in crosses in which selection was made for the transfer of prototrophic alleles from *R. meliloti* to *R. leguminosarum*, most of the rare progeny behaved as R-primes in which the appropriate *R. meliloti* prototrophic allele had been inserted into R68·45 (Johnston *et al.*, 1978*b* and *c*). For three such plasmids this was confirmed by showing that they were larger (by up to 45 Md) than the parental R68·45 (Johnston *et al.*, 1978*b*). These plasmids were used to confirm that genes with similar map locations in *R. meliloti* and *R. leguminosarum* were functionally equivalent. Each of eight R68·45 primes that were known to suppress particular mutations in *R. leguminosarum* were found to suppress mutations at corresponding positions in *R. melitoli* (Kondorosi *et al.*, 1980).

Plasmid primes in other bacteria have an enhanced ability to mobilize chromosomal genes located to one side of the DNA corresponding to that inserted in the plasmid. Such polarized transfer has been most studied in *E. coli* using F primes (see Low, 1972) but has also been seen in *Streptomyces* using an SCP1-prime (Hopwood and Wright, 1976). The R68·45 primes described above failed to mobilize the chromosome of *R. leguminosarum* at the region of the insert at enhanced frequencies, presumably because of lack of homology between the DNA of the two species (Johnston *et al.*, 1978*c*) but in *R. meliloti* they did exhibit enhanced, polarized transfer of genes close to the region of the inserted DNA. Interestingly, all R primes, carrying widely separated chromosomal regions, mobilized genes anti-clockwise of the insert (Kondorosi *et al.*, 1980); the significance of this finding is not clear. RP4 primes constructed *in vitro* by the insertion of restriction endonuclease fragments of the *R. meliloti* chromosome also promoted enhanced polarized chromosome transfer in this species but the polarity was clockwise for some RP4 primes and anti-clockwise for others (Julliot and Boistard, 1979).

The wide host range of P1 group plasmids allows *Rhizobium* genes to be examined for expression in other genera, by transferring such R primes to suitable recipients. Three R68·45 primes, each one of which suppressed all the *trp* alleles mapping in one of the *trp* regions of the *R. leguminosarum* chromosome, have been studied in some detail. By crossing these into different strains of *P. aeruginosa*, each carrying a mutation in a different *trp* gene, it was shown that one R prime suppressed *trpA*, *B* and *F* mutations, another *trp C* and *D* and the third *trp E* mutations. The expression of the *Rhizobium trp* genes, as measured by tryptophan-independent growth, was poor in the cases of the *trpA* and *trpB* genes but was apparently normal for the other genes. When these same plasmids were transferred to appropriate *E. coli* strains, with mutations in the various *trp* genes, no tryptophan-independent growth was detected (Johnston *et al.*, 1978*b*). It was possible to select a derivative of the plasmid that carried the *trpA*, *B* and *F* genes which could now express all three genes in *E. coli*, but we do not know the nature of the change in this derived plasmid, nor the nature of the block(s) to expression which it overcomes. Interestingly *E. coli* and *P. aeruginosa trp* genes can be expressed in *R. leguminosarum* (Johnston *et al.*, 1978*b*; Nagahari *et al.*, 1979), indicating that *E. coli* may be particularly fastidious when it comes to expressing "foreign" genes. If the case of the *Rhizobium trp* genes is typical, experiments designed to identify "symbiotic" gene products of *Rhizobium* by cloning such genes in *E. coli* and looking for expression may not be successful.

Lest it be thought that conjugal gene transfer in *Rhizobium* can be promoted only by sex-plasmids from other sources, it should be pointed out that some strains of *Rhizobium* contain transmissible plasmids and that these are capable of mobilizing the chromosome. Three plasmids, pRL1JI, pRL3JI and pRL4JI, were detected in three field isolates of *R. leguminosarum* on the basis that these strains made a bacteriocin whose production could be transferred at high frequences (*c.* 10^{-2}) to non-producing strains of *R. leguminosarum*, *R. trifolii* and *R. phaseoli* (Hirsch, 1979). All three plasmids could mobilize chromosomal alleles in *R. leguminosarum* but at low frequencies (10^{-8}-10^{-9}). Nevertheless linkage was found between pairs of alleles known to be linked in R68·45-mediated crosses.

Resident sex factors in *Rhizobium* may be rare. In a survey of 145 isolates of *R. phaseoli*, only six strains donated chromosomal genes to *R. leguminosarum* in crosses that would have detected gene transfer frequencies of 10^{-9} or higher (J. L. Beynon, personal communication). In one of these cases the donor *R. phaseoli* strain was shown to carry a transmissible bacteriocinogenic plasmid.

(iii) *Transduction*

Most studies on transduction have involved *R. meliloti*. In this species a high proportion (12 out of 21) of temperate phages were capable of transducing a *str* marker (Kowalski, 1970). One of these (L5) was used to transduce *leu*$^+$ into a leucine-requiring strain of *R. meliloti* which induced nodules incapable of fixing nitrogen; all Leu$^+$ transductants tested were able to fix nitrogen in the nodules, confirming that the leucine requirement was responsible for the symbiotic defect (Kowalski, 1974).

There has been a more detailed study of a specialised transducing phage, 16–3, which integrated specifically at a site near *cys–46* on the chromosome of *R. meliloti* strain 41 and could transduce this allele, but no others, at frequencies of 10^{-6} to 10^{-7} (Svab *et al.*, 1978). A high proportion of the Cys$^+$ transductants gave rise to lysates that could transduce Cys$^+$ at high frequencies (*c.* 10^{-2}), indicating that they were heterogenotes. Uniquely among the bacteriophages of *Rhizobium*, 16–3 has itself been subject to genetic investigation. Orosz *et al.* (1973) mapped a series of temperature-sensitive mutants and recently the DNA has been mapped using restriction nucleases (Dallmann *et al.*, 1979). These studies indicate that a large part of the DNA of this bacteriophage is either silent or codes for non-essential products since no mutants located in this region have been isolated.

A system of generalised transduction for *R. meliloti*, using a virulent bacteriophage, DF2, was described by Casadesus and Olivares (1979*a*). Transduction frequencies of many prototrophic alleles were similar (*c.* 10^{-6}). Co-transduction of pairs of alleles shown to be linked using R68·45 in conjugational crosses was also found. Bacteriophage anti-serum had to be added to protect the transductants from being killed by subsequent infection. Later, (Casadesus and Olivares, 1979*b*), this was circumvented by the use of a temperature-sensitive mutant of the bacteriophage, the transductants being grown at the non-permissive temperature.

Generalised transduction has also been demonstrated in *R. leguminosarum* using the virulent bacteriophage RL38JI (Buchanan-Wollaston, 1979), transductants being protected from subsequent infection by treating the lysate with ultra violet light to a survival of about 1 per cent prior to infection. Transduction of several chromosomal alleles occurred at frequencies of *c.* 10^{-6} per recipient. In three point crosses, three closely linked alleles, *rif*, *str* and *spc*, could be mapped and other pairs of alleles known to be closely linked could also be co-transduced. Furthermore RL38JI could transduce a number of alleles from *R. leguminosarum* to *R. trifolii* but transfer in the reverse direction was not observed. RL38JI can also transduce, apparently intact, plasmids at least as large as 80 Md, a result consistent with the large size of the phage DNA (*c.* 120 Md. Buchanan-Wollaston, 1979; A. V. Buchanan-Wollaston, personal communication). It

has also been used for transduction of parts of the large plasmids in *R. leguminosarum* into strains carrying such plasmids (Johnston *et al.*, 1978*a*; Buchanan-Wollaston *et al.*, 1980).

In the discussion above, two rather serious omissions may have been discerned. Firstly there was virtually no mention of the genetics of the slow-growing species such as *R. japonicum*, which reflects the paucity of reports on such species. Obviously their slow growth makes them less attractive for routine genetic manipulation, but there also appear to be difficulties in the isolation of auxotrophic mutants. Given the agronomic importance of some of their hosts it would be desirable obviously to be able to handle the slow-growing *Rhizobium* species genetically. P1 group plasmids have been transferred to *R. japonicum* but chromosomal recombination was not observed (Kuykendall, 1979).

The other point is that in the discussion above, mutants concerned with symbiotic functions were conspicuous by their absence. There are at least two reasons for this. One is fundamental—at least in some species, many "symbiotic genes" are not chromosomally located (see below)—but there is also a trivial reason: it is of course far easier to isolate and to map auxotrophic or antibiotic resistant mutations than symbiotic ones whose defective phenotypes can be determined only by testing on plants. This latter problem may be relieved in part by employing transposons as mutagens rather than the more commonly used chemical or physical agents. When an antibiotic resistance transposon inserts into a gene, it not only mutates that gene but also marks it with a readily selectable phenotype which facilitates subsequent mapping, both genetically and physically, since the insertion of an extra piece of DNA can be detected by hybridisation, restriction endonuclease mapping or electron microscopic methods.

Transposons can be introduced into *Rhizobium*. The introduction of bacteriophage Mu into the P1 group plasmid RP4 greatly decreases the frequency of inheritance of the plasmid when transferred from *E. coli* to *R. meliloti* (Boucher *et al.*, 1977). Van Vliet *et al.* (1978) took advantage of this finding to introduce the transposon Tn7 into *Agrobacterium*. Tn7 was inserted into RP4::Mu in *E. coli* and selection was made for transfer to *Agrobacterium* of the antibiotic resistance (streptomycin) specified by Tn7. Because the great majority of the intact plasmids fail to become established in *Agrobacterium*, this selection enriches for recipients in which Tn7 has transposed to recipient DNA. A similar approach was used to introduce the transposon Tn5 into *Rhizobium* (Beringer *et al.*, 1978*a*). Tn5, specifying kanamycin resistance (Berg *et al.*, 1975), was introduced into another P1 group plasmid (pPH1JI) into which bacteriophage Mu has been inserted. When kanamycin resistance was transferred from *E. coli* carrying this tripartite plasmid into *R. leguminosarum*, *R. trifolii* and *R. phaseoli*, about 0·5 per cent of the progeny were auxotrophic, and it was shown in the first two species that the auxotrophic mutation and the resistance were inseparable by transduction (Beringer *et al.*, 1978*a*). Some Tn5-induced symbiotic mutants of *R. leguminosarum* were shown to map to the chromosome (A. W. B. Johnston, unpublished observations) and others to resident plasmids (Buchanan-Wollaston *et al.*, 1980).

3. *Rhizobium* plasmids

(i) *Physical studies*

In addition to the genus *Rhizobium* the family Rhizobiaceae contains the genus *Agrobacterium*, members of which are also able to induce cell proliferation on host plants. All species of *Agrobacterium* apparently contain large plasmids having molecular weights greater than 10^8 (Van Larebeke *et al.*, 1974; Watson *et al.*, 1975; Sciaky *et al.*, 1978) which are associated with virulence properties of these species (see reviews by Schilperoort *et al.*, 1979; Moore *et al.*, 1979). Methods similar to those used for the isolation of large plasmids from *Agrobacterium tumefaciens* (Zaenen *et al.*, 1974; Currier and Nester, 1976; Ledeboer *et al.*, 1976) have been applied to *Rhizobium* and a wide range of large plasmids has been found in most of the strains examined (Nuti *et al.*, 1977; Casse *et al.*, 1979; Gross *et al.*, 1979). They were probably overlooked in earlier studies (Sutton, 1974; Klein *et al.*, 1975; Tshitenge *et al.*, 1975; Zurkowski and Lorkiewicz, 1976) because the simple cleared lysate procedure (Clewell and Helinski, 1969) used for plasmid isolation was unsuitable for the recovery of such large plasmids. Even with newer methods of plasmid isolation, the recovery of extremely large plasmids (more than 200 Md) can be sporadic.

Fig. 1 illustrates, on agarose gels, plasmids isolated from seven field isolates of *R. leguminosarum*. Each strain has a unique pattern of plasmids of different sizes, the molecular weights ranging from 50 to >200 Md. Since every strain contains more than one plasmid band, plasmid DNA represents at least 5-20 per cent of total chromosomal DNA, even if the plasmid copy number is low (1-3 per cell). The plasmids of *R. leguminosarum* strain 300 have been studied in some detail. Originally, three plasmid bands were detected in this strain (Prakash *et al.*, 1980), with molecular sizes of 100, 165 and 205 Md. However two additional plasmid bands of higher molecular weight can be seen in recent preparations and the smallest band is likely to contain two co-migrating plasmids since, in one derivative of strain 300 (strain 1062), it has apparently been resolved into two distinct bands, presumably as a result of a small deletion in one of them (P. R. Hirsch, personal communication; N. J. Brewin, unpublished observations).

(ii) *Mutagenesis*

What genetic functions are specified by these plasmids and under what conditions are they transferred between strains? In order to answer these questions we need mutants and identifiable genetic traits carried on the plasmids. Like any other DNA, plasmids are prone to mutagenesis by chemicals, by UV irradiation and by the insertion of transposons. In three out of six randomly chosen symbiotically defective mutants of strain 300 obtained after *N*-methyl-*N'*-nitro-N-nitrosoguanidine, the mutant phenotype was shown to be suppressed by the introduction of derivatives of a transmissible nodulation plasmid, pRL1JI, (Brewin *et al.*, 1980). These included non-nodulating (Nod^-) non-fixing (Fix^-) and temperature-sensitive fixing nodules; hence all these gene functions must be present on the

plasmid pRL1JI, although their position in strain 300 itself has not been determined.

A particularly interesting mutant, obtained after UV treatment, is strain 6007 (and its derivatives 6015 and 16015). This mutant, which is non-nodulating and non-reverting, has apparently suffered a substantial deletion in the 205 Md plasmid of strain 300 to give a new band of approximately 190 Md (fig. 2). Introduction of derivatives of pRL1JI into this strain resulted in suppression of the mutant phenotype (Johnston *et al.*, 1978*a*). Moreover when mutagenised derivatives of pRL1JI were used as donors (Buchanan-Wollaston *et al.*, 1980) some Fix$^-$ transconjugants were obtained, implying that the deletion in strain 6007 spans both *nod* and *fix* genes.

The most useful method for the mutagenesis of plasmids involves the insertion of transposons (Beringer *et al.*, 1978*a*). This can be accomplished in several ways. Firstly, since plasmid DNA can represent as much as 10 per cent of total DNA, it is not unreasonable to screen random samples of transposon-containing clones either genetically for the existence of transmissible drug resistance (Johnston *et al.*, 1978*a*) or physically by the hybridization of radioactively labelled transposon to plasmids separated by gel electrophoresis. Alternatively, where a strain is known to contain a highly transmissible plasmid, it is possible to enrich specifically for insertions into this plasmid (Buchanan-Wollaston *et al.*, 1980). The technique is to use, as a donor, a strain containing the transposon inserted into a mapped chromosomal gene, and then to select for transfer of transposon-specified drug-resistance to another strain. The only way such a transfer can arise is by prior transposition of the transposon into the transmissible plasmid. Once transposons have been introduced into plasmid DNA, they can subsequently be transduced to regions of DNA homologous to that flanking the transposon in different plasmids in different strains (Brewin *et al.*, 1980; (Buchanan-Wollaston *et al.*, 1980) some Fix- transconjugants were when the plasmids are large compared to the size of the DNA in the transducing phage.

Variants which have been cured of a plasmid altogether are important in elucidating the role of that plasmid. Zurkowski and Lorkiewicz (1978) obtained non-nodulating mutants of *R. trifolii* after incubation for seven days at 35°C. Similarly Casse *et al.* (1979) after heat treatment of a strain of *R. leguminosarum*, obtained a non-nodulating mutant that lacked one of the three large plasmids of the parent strain. Higashi (1967) found that the ability of *R. trifolii* to nodulate clover was lost at high frequency following treatment by acridine orange, an agent known to cause curing of plasmids in other bacteria. However, neither heat-treatment nor acridine orange is universally successful, and to our knowledge no strain of *Rhizobium* exists that has been cured of all its plasmids.

(iii) *Genetic functions located on plasmids*

Although the available information is still very limited, it is now possible to give some idea of the kinds of gene functions carried on the plasmids of *Rhizobium*.

(a) Medium *bacteriocin production*. Hirsch (1979) identified three transmissible bacteriocinogenic plasmids of *R. leguminosarum*, each from a

Plate I

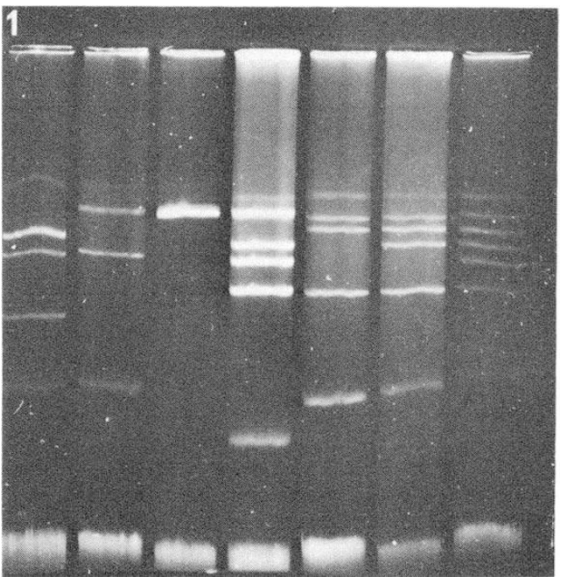

FIG. 1.—Separation of large plasmids from lysates of seven field isolates of *R. leguminosarum* (G. Hombrecher, unpublished result)

Log phase cells were lysed with pronase/SDS and the pH raised to 12·4 and then lowered to 8·0. Chromosomal DNA was precipitated by the addition of NaCl (to a final concentration of 1 M) and the remaining, plasmid-enriched, DNA was precipitated with polyethylene glycol, redissolved in buffer and analysed by electrophoresis on 0·7 per cent agarose gels in Tris borate buffer, pH 8·3.

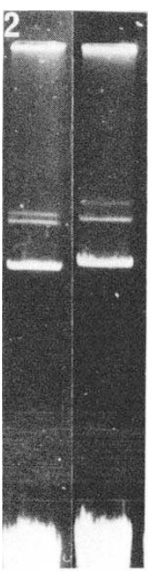

FIG. 2.—Analysis on agarose gels of plasmids isolated from strains 6015 (left track) and 300 (right track). Note that in strain 6015 the largest visible plasmid is smaller than the corresponding one in strain 300 (P. R. Hirsch, unpublished results).

different field isolate. These plasmids were designated pRL1JI, pRL3JI and pRL4JI. The bacteriocins specified by these plasmids were apparently related because the presence of any one of these plasmids in a strain conferred resistance to bacteriocin(s) produced by either of the other two. In each case the specified bacteriocin was of *medium* size, *i.e.*, it diffused about 5 mm in agar plates but could not cross a dialysis membrane. Moreover there was evidence of DNA sequence homology between the three plasmids in the region specifying bacteriocin production: a Tn5 insertion into genes specifying *medium* bacteriocin production on pRL1JI could be transduced into strains carrying pRL3JI or pRL4JI with concommittent loss of *medium* bacteriocin production from these plasmids (Brewin *et al.*, 1980).

(b) Small *bacteriocin repression*. The introduction of any of the bacteriocinogenic plasmids pRL1JI, pRL3JI or pRL4JI into *R. leguminosarum* strain 300 (or other strains) which normally produces a *small* bacteriocin (diffusible through dialysis membrane) results not only in the expression of *medium* bacteriocin production but also in the repression of *small* bacteriocin production. The possibility that this reflects elimination of a plasmid specifying *small* bacteriocin which is incompatible with the incoming *medium* bacteriogenic plasmid is unlikely for two reasons: none of the plasmid bands seen in lysates of strain 300 was lost following the introduction of the bacteriocinogenic plasmid, and curing of a derivative of strain 300 containing pRL1JI::Tn5 results in recovery of the ability to produce *small* bacteriocin following loss of the *medium* bacteriocinogenic plasmid (P. R. Hirsch and A. W. B. Johnston, unpublished observations). Thus there is good evidence that each of the bacteriocinogenic plasmids pRL1JI, pRL3JI and pRL4JI carries a gene specifying a repressor for *small* bacteriocin production. Moreover this gene must be distinct from that specifying production of *medium* bacteriocin because a transposon insertion which eliminates the latter function has no effect on the former (Johnston *et al.*, 1978*a*).

(c) *Nodulation ability*. The derivative of *R. leguminosarum* strain A171 that has been cured of its smallest plasmid fails to nodulate peas (Casse *et al.*, 1979). Similarly, heat-treated *R. trifolii* strains were enriched for non-nodulating strains (Zurkowski and Lorkiewicz, 1978). In *R. leguminosarum* strain 300 the mutant strain 6007, which carries a deletion in the 205 Md plasmid, is Nod^- and is restored to Nod^+ Fix^+ by the introduction of derivatives of the plasmid pRL1JI (Johnston *et al.*, 1978*a*), but not of the apparently related bacteriocinogenic plasmids pRL3JI and pRL4JI. Another *R. leguminosarum* plasmid, pRL5JI, derived from the field isolate TOM (Lie and Winarno, 1979), is also capable of restoring a Nod^+ Fix^+ phenotype to derivatives of 6007 (N. J. Brewin, unpublished observations).

(d) *Host range specificity*. Higashi (1967) demonstrated transfer of clover infectivity from *R. trifolii* to *R. phaseoli*. More recently transfer of derivatives of the plasmid pRL1JI from *R. leguminosarum* to strains of *R. trifolii* and *R. phaseoli* was shown to confer to transconjugant clones the ability to nodulate peas in addition to their normal hosts, *Trifolium* and *Phaseolus* respectively (Johnston *et al.*, 1978*a*). Even within the pea cross-inoculation group, certain restrictions on host range are known; the *R. leguminosarum* strain TOM is unusual in that it is able to nodulate a primitive pea cultivar (Afghanistan) which cannot be nodulated by European field isolates of *R. leguminosarum* (Lie and Winarno, 1979). The

nodulation plasmid pRL5JI, when transferred from strain TOM into a derivative of strain 300, transfers the particular host range properties of the donor strain TOM (N. J. Brewin, unpublished observations).

(e) *Nodule function.* The fact that 14 out of 160 of the mutagenised derivatives of the nodulation plasmid pRL1JI (Buchanan-Wollaston, *et al.*, 1980), when transferred into the plasmid deletion mutant 6015, resulted in the production of Fix^- nodules on peas indicates that both Nod and Fix functions are normally present on pRL1JI, and incidentally that these same functions are also normally present on the 205 Md plasmid of strain 300 but are absent from this plasmid deletion mutant. Similarly, the fact that the introduction of pRL5JI into strain 16015 resulted in the production of Fix^+ nodules by transconjugant clones argues that pRL5JI also carries all the Nod and Fix gene functions that are absent from strain 16015.

(f) *Nitrogenase.* It is not known what gene products contribute to the Fix^+ phenotype of the functional root nodule but clearly nitrogenase itself is an essential component. Recently it has been shown that there is sufficient homology between DNA specifying some of the nitrogenase (*nif*) components from *Rhizobium* and *Klebsiella* to allow cross-hybridisation to be detected using radioactive cloned *nif* DNA from *Klebsiella* and a restriction digest of *Rhizobium* DNA, separated by gel electrophoresis (Ruvkun and Ausubel, 1980). Moreover, this hybridisation was also detected to purified *R. leguminosarum* plasmid DNA (Nuti *et al.*, 1979). This is strong evidence that at least some of the structural genes for *R. leguminosarum* nitrogenase are plasmid-borne.

The reported interspecific transfer of nitrogen-fixing ability (Nif^+) from strains of *R. trifolii* containing P1 group R plasmids into an avirulent strain of *Agrobacterium tumefaciens* and also into a non-fixing strain of *Klebsiella aerogenes* (Dunican and Tierney, 1974; Stanley and Dunican, 1979) is most easily explained if the *Rhizobium nif* genes are present on a plasmid that is co-transferred at high frequency with the R plasmid. Similarly the reported transformation of a Nif^- mutant of *Azotobacter vinelandii* to a Nif^+ phenotype by DNA from *R. trifolii* (Bishop *et al.*, 1977) and from *R. japonicum* (Maier *et al.*, 1978*a*) could have involved transformation by a whole plasmid. However, in none of these cases has the appearance of a new plasmid species in the recipient been described.

(g) *Cell wall polysaccharide.* A Nod^- derivative of *R. leguminosarum* strain LPR1705 which had been cured of its smallest plasmid has a "rough" colony morphology which presumably reflects an alteration in the composition of its extracellular polysaccharide (Prakash *et al.*, 1980). The reported transformation of an *A. vinelandii* Nif^- mutant to a Nif^+ phenotype with DNA isolated from *R. trifolii* (Bishop *et al.*, 1977) was associated with the transfer of clover lectin-binding ability into the transformants; possibly this genetic determinant was linked to *nif*. Similarly when *R. japonicum* DNA was used, some of the Nif^+ transformants of *Azotobacter* had acquired the O-antigen-related polysaccharide that was present on the cell surface of the nodulating *R. japonicum* strain (Maier *et al.*, 1978*a*).

(h) *Pigment production.* Old colonies of *R. phaseoli* on agar plates containing tryptone and yeast-extract, or on minimal medium plus tyrosine, produce a dark brown pigment, which may be melanin. Pigment production, the ability to form effective nodules on *Phaseolus* beans, and a plasmid are lost simultaneously from a strain of *R. phaseoli* (J. L. Beynon, personal

communication). This suggests strongly that pigment production is controlled by the same plasmid that determines nodulation ability for *Phaseolus* beans, although the physiological importance of this pigment is unknown.

(i) *Transfer functions.* The bacteriocinogenic plasmids pRL1JI, pRL3JI and pRL4JI are all transferred between strains of *R. leguminosarum* at high frequencies, *c.* 10^{-2} per recipient (Hirsch, 1979). The transfer of the 205 Md nodulation plasmid of strain 300 to strain 16015 must be lower than 10^{-6} per recipient (Brewin *et al.*, 1980). The smallest (100 Md) pair of plasmids in strain 300 derivatives can be transferred with derivatives of the bacteriocinogenic plasmid pRL1JI at frequencies of (1-10 per cent (N. J. Brewin, unpublished observations and J. L. Beynon, personal communication). These data illustrate the range of transmissibility among *Rhizobium* plasmids. The observed frequencies of transfer may depend on properties of the donor and recipient strains as well as on transfer functions specified by the plasmid itself. Presumably, as transposon insertions into *Rhizobium* plasmids become available, the transmissibility of individual plasmids will become easier to measure.

(j) *Incompatibility.* The plasmids pRL1JI, pRL3JI and pRL4JI appear to belong to the same incompatibility group: they cannot apparently co-exist within the same cell (Brewin *et al.*, 1980). None of these plasmids is incompatible with any of the resident plasmids of strain 300 (P. R. Hirsch, personal communication) now with another nodulation plasmid, pRL5JI, which can co-exist both with derivatives of pRL1JI and with the resident plasmids of strain 300 (N. J. Brewin, unpublished observations).

When transferred to a strain of *R. phaseoli* the plasmid pJB5JI (pRL1JI::Tn5) is compatible with both of the resident *R. phaseoli* plasmids; however in the rare transconjugants that had also received the smallest plasmid from the 300 derivative, 1062, the smaller *R. phaseoli* plasmid was always elminated (J. L. Beynon, personal communication). This *R. phaseoli* plasmid determines pigment production and the formation of functional nodules on *Phaseolus* beans (see above), but the plasmid from strain 1062, which apparently belongs to the same incompatibility group, has no known function. Clearly the relationship between nodulation plasmids and incompatibility groups is far from simple.

Although certain plasmids may co-exist within a *Rhizobium* strain when grown *ex planta*, they may not be able to do so within the root nodule. For example the introduction of the plasmid pJB5JI into a strain of *R. phaseoli* did not eliminate any of the resident plasmids from this strain when transconjugants were grown on Petri dishes. However pJB5JI confers nodulation ability for peas, and when clones were recovered from pea nodules they were all found to have suffered deletion or elimination in one of the *R. phaseoli* plasmids (J. L. Beynon, unpublished observations). These results suggest that there may be functional interference between genes specified by the nodulation plasmid pJB5JI and corresponding genes present on the *R. phaseoli* plasmid. This interference prevents the formation of nodules on peas and only those derivatives that have suffered spontaneous deletions or loss of the *R. phaseoli* plasmid are able to form nodules. This type of interaction could be described as "physiological incompatibility".

(iv) *Evidence for genetic rearrangements*

The bacteriocinogenic plasmids pRL3JI and pRL4JI, when transferred into the non-nodulating *R. leguminosarum* strain 16015, only restored nodulation ability (Nod^+) at a frequency of 10^{-3} per plasmid transfer (Brewin *et al.*, 1980). The same was true for derivatives of these plasmids containing the transposon Tn5, namely pVW3JI and pVW5JI. However, when the nodulating transconjugants were subsequently used as donors, it was found in more than half the cases that Nod^+ was now transferred at the same high frequency as kanamycin resistance, such that five out of five of the transconjugant clones nodulated peas. In other words, by selecting for transfer of Nod^+, derivatives of pRL3JI or pRL4JI were obtained which had acquired the ability to transfer Nod^+ at high frequency in subsequent crosses (Brewin *et al.*, 1980), presumably as a result of recombination.

(v) *Comparison with other members of the Rhizobiaceae*

In both *Agrobacterium*, and *Rhizobium*, large plasmids have been shown to determine infectivity and host range (Van Montagu and Schell, 1979). However, there is no evidence that part of any *Rhizobium* plasmid might be transferred to the genome of the host plant in a manner analogous to the T-DNA of *A. tumefaciens*. In addition there is no evidence that *Rhizobium* induces the formation in its host plant of substances like octopine, nopaline or agropine for which large plasmids might carry catabolic functions (Schilperoort *et al.*, 1979), although the occurrence of an unidentified amino compound in soybean root nodules induced by an ineffective field isolate might conceivably be a pointer in this direction (Werner *et al.*, 1980).

4. The use of mutants for the genetic analysis of symbiotically important genes

The genetic analysis of metabolic pathways in microorganisms has been facilitated by the availability of mutant strains defective in different stages of the pathway, or in its regulation. It is logical therefore to attempt to analyse the role of *Rhizobium* in the symbiosis by the same procedures.

(i) *Naturally occurring symbiotically-defective strains*

Two symbiotic phenotypes that are readily recognizable are the ability to form a nodule (Nod^+) and the ability of the rhizobia to fix nitrogen within a nodule (Fix^+). Traditionally these phenotypes have been designated infective (Inf^+) and effective (Eff^+) respectively. A number of naturally-occurring Nod^- *Rhizobium* strains have been reported, but there has always been doubt about whether they are mutants of the relevant *Rhizobium* species since the only absolute taxonomic criterion for classification of a bacterium as *Rhizobium* is its ability to induce nodules on the roots of legumes (Kleczkowska *et al.*, 1968).

Fix^- rhizobia can be isolated from root nodules arising on field-grown legumes and several are available in culture collections but they have several disadvantages for genetic studies of symbiotically important genes. One is

the absence of the wild-type parents for comparison but there is a more fundamental problem. Rhizobia can form nodules on legumes belonging to the wrong cross-inoculation group (Wilson, 1944) but nitrogen fixation is never observed; examples are the nodulation of subterranean clover (*Trifolium subterraneum*) (Hepper and Lee, 1979) and *Phaseolus vulgaris* (J. L. Beynon, personal communication) by *R. leguminosarum.* In each case, isolation of bacteria from these nodules would produce strains which were apparently Fix$^-$ derivatives of *R. trifolii* and *R. phaseoli* respectively but are in fact normal strains of another species.

(ii) *Symbiotic properties of auxotrophic and resistant mutants*

The plant tests required to examine the symbiotic phenotype of *Rhizobium* strains are time-consuming and expensive. Therefore, there has been considerable interest in the analysis of auxotrophic or resistant mutants which may coincidentally have symbiotically defective phenotypes. This approach has two significant advantages: the initially selected phenotype is relatively simple to obtain, especially in the case of resistant mutants, and if mutants are found to be symbiotically defective, there is a reasonable chance that the symbiotic defect can be explained from an analysis of the defect causing the auxotrophic or resistant phenotype.

There are a number of disadvantages which should be considered, even though they do not invalidate the use of the method:

(a) The pleiotropic phenotype may be due to multiple mutations, especially after mutagenesis.
(b) For auxotrophic mutants it is difficult to determine whether the metabolite in question is essential for the establishment of nitrogen-fixing nodules, or whether the plant is unable to provide enough for normal growth of the rhizobia.
(c) Because selection is for pleiotropic mutants, mutations in genes solely involved in symbiotic properities will be missed.

(a) *Phage resistance.* Probably the first reports of the indirect selection of symbiotically defective mutants were by Nobbe and Hiltner, and Frank in the 1890s (Kleczkowska, 1950) who reported that prolonged culture on gelatin produced strains which were less effective at nitrogen fixation within nodules. The first characteristic specifically chosen to provide a direct selection for symbiotically defective mutants was phage resistance (Krasilnikov, 1941*b*; Kleczkowska, 1950, 1965). More than half the phage resistant mutants of some strain of *R. trifolii* were Fix$^-$ (Kleczkowska, 1950, 1965). It is tempting to think that the phage resistance was due to alteration in cell walls and that this prevented the rhizobia from differentiating into nitrogen fixing bacteroids (Dénarié *et al.*, 1976). Unfortunately studies comparing cell walls of wild-type and phage-resistant mutants of *Rhizobium* (Atkins and Hayes, 1972; Zajac *et al.*, 1975) have been done without reference to possible symbiotic properties of the resistant mutants.

(b) *Drug resistance.* Mutation to drug resistance can also be due to changes in the outer layers of microbial cells. Studies of the symbiotic properties of such mutants have been reviewed by Dénarié *et al.* (1976). Schwinghamer (1967) first noted a correlation between resistance to antibiotics "known" to inhibit cell wall synthesis and symbiotic defectiveness,

and this has been discussed by Dénarié *et al.* (1976) and Pankhurst (1977). The only detailed biochemical studies of cell walls from resistant mutants are those of McKenzie and Jordan (1970, 1972) and Yu and Jordan (1971). They studied viomycin-resistant mutants of *R. meliloti*, which were shown to accumulate phospholipids in their cell walls, to have altered levels of cations, and altered cation exchange properties. These mutants were Fix^- and did not form bacteroids (Hendry and Jordan, 1969). Unfortunately, further studies of rhizobia presumed to be resistant to phages or drugs through alterations in cell walls have not been reported in detail. Bacteroid formation appears to be a prerequisite for nodule nitrogen fixation by most fast-growing *Rhizobium* species and this involves cell wall changes leading to an increased volume, shape and wall structure (Dart, 1975). Further analysis of resistant mutants of the type described above would, therefore, appear to be desirable.

Two other types of drug resistance which might also be expected to give rise to a symbiotic defect on the basis of the known modes of resistance in other microorganisms are those to rifampicin and aminoglycoside antibiotics. Rifampicin resistance can be due to changes in the permeability of bacteria or to alterations of the RNA polymerase so that it no longer binds the drug (Marshall and Gillespie, 1972; Chater, 1974). In *Bacillus subtilis* a proportion of rifampicin-resistant RNA polymerase mutants are sporulation deficient (Sonenshein and Losick, 1970) which implies that changes in the RNA polymerase may be important in allowing the normal differentiation that leads to spore formation. Pankhurst (1977) and Pain (1979) reported that a proportion of rifampicin-resistant mutants of *Rhizobium* were Fix^-; and, for *R. leguminosarum*, Pain (1979) found that of eight antibiotics tested, rifampicin was the only one for which resistant mutants were symbiotically defective. Whether the Fix^- rifampicin-resistant mutants were due to permeability changes or defects in the RNA polymerase remains to be determined. If it is due to a modification of the RNA polymerase, as proposed by Pain (1979), this may suggest a similar role for RNA polymerase in the differentiation of *Rhizobium* as in *B. subtilis* during sporulation.

Mutational resistance to aminoglycoside antibiotics (such as kanamycin and neomycin) has been found to be correlated with defects in the coupling of oxidative phosphorylation with ATP synthesis in *E. coli* (Cox and Gibson, 1974) and selection for neomycin-resistance is a common method for isolating uncoupled (Unc^-) mutants (Kanner and Gutnick, 1972). Skotnicki and Rolfe (1979) isolated neomycin-resistant mutants of *R. trifolii* strain T1 which were unable to grow on succinate as sole carbon source. The five mutants studied in detail had reduced ATPase activity and altered growth properties and were unable to synthesize ATP, all characteristics shown by *E. coli unc* mutants. Revertants which were able to utilize succinate as sole carbon source became phenotypically Unc^+ and neomycin sensitive, indicating that the neomycin-resistance and Unc^- phenotype were due to mutation of the same gene. Surprisingly, all five *unc* mutants were Fix^+ and the bacteroids of two of these were found to have about 70 per cent of the ATPase activity of the wild-type parent strain. This ATPase activity was apparently not correlated with reversion, because no succinate-utilizing bacteria were isolated from bacteroid preparations (Skotnicki and Rolfe, 1979). It appears, therefore, that bacteroids acquire a new ATPase activity

synthesized perhaps as a result of bacteroid differentiation (see Skotnicki and Rolfe, 1979). While it is logical to pursue studies of drug-resistant rhizobia, it should be remembered that our understanding of the mode of action of most antibiotics in bacteria is limited, and we know even less about the biochemical changes in drug-resistant mutants. Changes in permeability are commonly attributed to resistance mutations which do not appear to alter the antibiotic target site, but in most cases this is not established.

(c) *Antimetabolite-resistant and auxotrophic mutants.* These two classes of mutant are best described together because, surprisingly, a large proportion of antimetabolite-resistant rhizobia are partially auxotrophic (especially D-alanine- and D-histidine-resistant mutants (Schwinghamer, 1969)). Some of these auxotrophic, antimetabolite-resistant mutants are symbiotically defective (Schwinghamer, 1969). However, the use of antimetabolite-resistance as an initial selection for auxotrophic and/or symbiotically-defective mutants is limited by the extremely pleiotropic nature of the mutations (table 2). The riboflavin-requiring, D-histidine-resistant, Fix^- mutant number 15 in Table 2 has been studied in some detail. The addition

TABLE 2

Phenotype of D-histidine-resistant, auxotrophic mutants of R. trifolii *strain T1 (from Schwinghamer, 1969)*

	Compounds stimulating growth		
Clone number	Moderate stimulation	Heavy stimuation	Symbiotic phenotype
7	none	none	Fix^-
10, 13	inositol	—	Partially Fix^-
15	—	riboflavin	Fix^-
20, 21, 22	choline, adenine, xanthine	histidine	Partially Fix^-
26	pantothenate, inositol, serine	thiamine	Fix^-
28	pantothenate	thiamine	Fix^-
30	cystine	thiamine	Fix^-

of riboflavin to red clover (*Trifolium pratense*) nodulated with this mutant restored nitrogen fixation (Schwinghamer, 1970), and the mutant could form Fix^+ nodules on some varieties of subterranean clover (*T. subterraneum*) while it formed only Fix^- noldules on other varieties and on red clover (Pankhurst *et al.*, 1974). This was correlated with the different levels of flavin in the tissues of the plants, the Fix^+ varieties having about five times the amount of the other varieties. Riboflavin was required for the rhizobia to differentiate into bacteroids (Pankhurst *et al.*, 1972) and when it was removed after they had formed, nitrogen fixation continued. Thus, the differentiation of *R. trifolii* depended on a high level of riboflavin, while growth within the nodule and nitrogen fixation as bacteroids were supported by lower levels.

As well as showing that riboflavin was important for nodule development, this study very clearly demonstrated that the symbiotic phenotype of auxotrophic mutants can be conditional, depending on the ability of the host plant to provide the essential metabolite. The effect of the choice of host-plant on the symbiotic phenotype of auxotrophic mutants has been particularly noticeable with our studies of *R. trifolii* on white clover (*T.*

repens) (A. W. B. Johnston, unpublished observations) and *R. leguminosarum* on peas (*Pisum sativum*)(Pain, 1979). For *R. leguminosarum* strain 300, adenine auxotrophs were unique amongst a wide range of auxotrophic mutants in being symbiotically defective, whereas a very high proportion of *R. trifolii* auxotrophic mutants for a wide range of requirements were defective. This difference may have been due to the greater size of the pea plants which enabled them to provide amino acids or pyrimidines at concentrations adequate for normal nodule development.

Dénarié *et al.* (1976) discussed the effect of auxotrophy on *Rhizobium*, and for *R. meliloti* showed that uracil-, leucine- and adenine-requiring mutants (and some isoleucine-valine-requiring mutants) were symbiotically defective. It is difficult to determine the role of leucine and uracil in nodule formation since auxotrophic mutants for both these substances form Fix^+ nodules on peas (Pain, 1979). However, there appears to be a strong correlation between the requirement for adenine and symbiotic deficiency (Pankhurst and Schwinghamer, 1974; Dénairé *et al.*, 1976; Pain, 1979). A possible role for adenine during nodule formation is as a precursor for cytokinins, which are N-6 substituted derivatives of adenine. Rhizobia have been reported to produce small amounts of cytokinins (Phillips and Torrey, 1970) and hence adenine auxotrophs may be unable to produce adequate amounts. However this type of argument does not appear to apply to the production of indole-acetic acid, a tryptophan derivative which is another hormone produced by rhizobia (Dullaart, 1970). All 20 Trp^- auxotrophs of *R. leguminosarum* studied by Pain (1979) and one of *R. meliloti* studied by Dénairé *et al.* (1969) formed apparently normal nitrogen fixing nodules.

A major difficulty encountered in the study of the symbiotic properties of auxotrophic mutants of *Rhizobium* is reversion to prototrophy. Prototrophic revertants isolated in laboratory media are valuable for correlating the symbiotic phenotype with the auxotrophic requirement (Scherrer and Dénairé, 1971). Unfortunately, in many axenic nodulation test procedures, both the volume of medium and the provision of nutrients by the plant are great enough to allow large populations of rhizobia (greater than 10^8 per plant) to develop. Many auxotrophic mutants have reversion rates of about $10^{-9} - 10^{-7}$ and prototrophic revertants can therefore arise. Since they have a growth advantage in the nutritionally poor medium they can produce large populations. Scherrer and Dénarié (1971) and Ludwig and Signer (1977) reported the isolation of prototrophs from nodules of alfalfa (*Medicago sativa*) plants inoculated with auxotrophic derivatives of *R. meliloti* and Pain (1979) found that the majority of nodules on peas formed by most auxotrophic mutants of *R. leguminosarum* contained prototrophic revertants. A good example of this effect is shown in table 3.

The data in table 3 were obtained from pea plants grown in about 200 ml of a nitrogen-free mineral salts medium. Strain 897 (Phe^-Trp^-Str-r) forms Fix^+ nodules which are indistinguishable from those formed by the wild-type parent strain 300 and all bacteria isolated from the nodules are Phe^-Trp^-Str-r. However, the Phe^- parent, 603, forms nodules which usually contain Phe^+ revertants. A similar difference is observed with the Nod^- strain 6008 and the Phe^+ derivative 6008(1); no nodules are formed by 6008, whereas 6008(1) forms nodules on peas containing Trp^+ revertants. These results indicate that when only one auxotrophic mutation is present the rare revertants that arise can grow rapidly in the nutritionally

TABLE 3

Nodulation by auxotrophic derivatives of R. leguminosarum *strain 300*

Strain	Markers	Symbiotic phenotype	Properties of bacteria isolated from nodules
300	prototrophic	Fix^+	All nodules prototrophic
603	*phe-1*	Fix^+	Some nodules Phe^-, rest prototrophic
897	*phe-1 trp-12 str-37*	Fix^+	All nodules Phe^-, Trp^- Str-r
6008	*phe-1 trp-12 str-37 nod-6008*	Nod^-	—
6008(1)	Phe^+ derivative of 6008	Nod^-*	All nodules prototrophic, Nod^+, Str-r

* Assumed phenotype on the basis that it is a spontaneous Phe^+ derivative of strain 6008.

poor medium, whereas for doubly auxotrophic strains, even if one allele reverts, the presence of the remaining nutritional requirement prevents the rapid population rise. For strain 603 these then compete for nodule formation with the auxotrophic parent, and in the case of strain 6008(1) these Trp^+ revertants grow to a population where Nod^+ revertants arise. Reversion within nodules is unlikely because the reversion rate of the mutations is about 10^{-8} and nodules containing 10^8 or more rhizobia are unusual. Presumably the auxotrophs (such as strain 603) initiate nodules before the population has reached a level where prototrophs arise. Thus it would appear that even though prototrophs are not present initially, the growth of nodules formed by them is faster (or favoured by the plant) and few early infections are able to proceed to a point where nodules were produced.

A number of important points arise from these observations.

(a) Data for the symbiotic abilities of *Rhizobium* mutants are only reliable if isolates from nodules are tested for the retention of relevant mutations.

(b) The reports (Maier and Brill, 1976; Beringer *et al.*, 1977) of screens mutagenized cultures of *Rhizobium* for symbiotically defective mutants have probably underestimated the number of mutants, particularly Nod^-.

(c) Because rhizobia grow in the rhizosphere and the medium used for plant tests it is necessary to restrict their growth rate so that mutant phenotypes can be expressed before revertants arise. The use of multiply auxotrophic strains (like 897) or other methods of limiting the population are needed. However, these will impose their own constraints on the behaviour of mutants. Expression of the mutant phenotype in different auxotrophic backgrounds or growth conditions would be needed to clarify the role of the mutant gene.

Despite these limitations, several symbiotically defective rhizobia have been isolated after mutagenic treatments (Maier and Brill, 1976; Beringer *et al.*, 1977) Surprisingly, about half the Fix^- mutants of *R. leguminosarum* isolated by Beringer *et al.* (1977) were temperature-sensitive; normal Fix^+ nodules were produced at 15° but those formed at 25° were Fix^-. The rhizobia grew apparently normally on complete or defined media even at 28°. Several other temperature-sensitive symbiotically defective mutants of *R. leguminosarum* have been identified in our laboratory recently. For

example, some mutants able to grow in the presence of high levels of succinate are Fix^-. One is deficient in the uptake of succinate and cannot grow on low levels of succinate; revertants able to grow on low levels of succinate are Fix^+ and sensitive to high levels. Occasionally pink nodules are formed by the resistant mutant; bacteria isolated from one of these nodules were found to be Fix^+ at 15° but Fix^- at 25° (N. J. Brewin, unpublished results). Some rifampicin-resistant mutants which were Fix^+ under normal screening procedures are Fix^- or only partially Fix^+ at 25°. It might appear to be useful to have access to so many temperature-conditional mutants, because of the ease with which the functioning of the rhizobia during nodule development could be manipulated, but temperature-sensitivity may be an indication that general "fitness" is very important for normal nodule development and nitrogen fixation. The argument for this idea is similar to that for the auxotrophic mutants; the mutant rhizobia may be still capable of growth and causing infection when stressed (by, for example, low levels of a required metabolite such as riboflavin) but are unable to undergo steps required for differentiation so that a nitrogen-fixing nodule is produced. Interestingly, the two temperature-sensitive symbiotically-defective mutants of *R. leguminosarum* which we have mapped are chromosomal and may represent mutants in genes whose function is not required for growth on our minimal medium, or whose function is insufficiently impaired to prevent growth. Perhaps for *R. leguminosarum*, at least, genes whose functions are solely required for the development of a successful symbiosis are plasmid-borne, while those required also by free-living rhizobia are chromosomal.

(d) *Mutational studies of carbon and nitrogen metabolism.* The rhizobia within root nodules depend on the plant as a source of energy and as an eventual sink for fixed nitrogen. Free-living rhizobia can utilize a wide range of carbohydrates for growth (Graham, 1964*b*; Skotnicki and Rolfe, 1977; Ronson and Primrose, 1979), but there is still doubt about the main source(s) of energy for bacteroids within the nodule. In an attempt to resolve this problem Ronson and Primrose (1979) isolated mutants of *R. trifolii* which were unable to grow on most hexose sugars. These included mutants deficient in glucokinase (*glc*), fructose uptake (*fup*), the Entner-Doudoroff pathway, and pyruvate carboxylase. All mutants (including a *glc fup* double mutant) tested on clover plants formed nitrogen fixing nodules and no reverants were found in any nodule tested. A phosphoglucose isomerase mutant of *R. meliloti* isolated by Arias *et al.* (1979) also formed nitrogen fixing nodules, though it was less efficient than the wild-type parent. The results of both these papers imply that tricarboxylic acid cycle intermediates may be the major source of energy for bacteroids and this conclusion is reinforced by the preliminary report (Duncan and Fraenkel, 1979) that an α-ketoglutarate dehydrogenase (*kgd*) mutant of *R. meliloti* formed Fix^- nodules on alfalfa plants. This *kgd* mutant was unable to utilize arabinose, acetate and, surprisingly, pyruvate. However further studies are need to determine whether one, or a limited number, of energy sources are utilized by bacteroids.

Another source of energy available to rhizobia within nodules is the hydrogen which is evolved by nitrogenase during nitrogen fixation (Evans *et al.*, 1977). Some rhizobia have uptake hydrogenases which, when hydrogen is added to the atmosphere, are capable of supporting nitrogenase activity

(Emerich *et al.*, 1979) and, for *R. japonicum*, to allow autotrophic growth (Hanus *et al.*, 1979). To determine the importance of uptake hydrogenases in the efficiency of nitrogen fixation within the nodule, Maier *et al.* (1978*b*) isolated mutants of *R. japonicum* which were unable to use hydrogen. These mutants produced nodules which evolved hydrogen, unlike the parent, and showed lower acetylene reduction rates, which suggests that recycling of hydrogen by the parent may have been responsible for its greater acetylene reduction ability.

Studies of nitrogen metabolism by rhizobia have been restricted by the inability to obtain nitrogen fixation in the absence of nodule formation. *Ex planta* nitrogen fixation was demonstrated in 1975 (Kurz and LaRue, 1975; McComb *et al.*, 1975; Pagan *et al.*, 1975), but has not yet been reliably established for fast-growing rhizobia. Because of the difficulties associated with genetic studies of slow-growing rhizobia there is little knowledge of the genetics of nitrogen fixation and nitrogen metabolism in these bacteria. However Ludwig and Signer (1977) isolated a glutamine synthetase mutant of a cowpea *Rhizobium*, strain 32H1, which had no nitrogenase activity *ex planta* and formed Fix^- nodules. Thus, although it appears from other studies (O'Gara and Shanmugam, 1976) that nitrogen fixing rhizobia cannot accumulate ammonia, glutamine synthetase plays a role in regulating nitrogen fixation. This has also been demonstrated by Kondorosi *et al.* (1977*b*) who found that a glutamine synthetase mutant of *R. meliloti* formed Fix^- nodules. Kiss *et al.* (1979) also studied mutants of *R. meliloti* affected in nitrate reduction. They mapped in four genes at different sites on the chromosome and all formed Fix^+ nodules. These included two genes involved in the formation of an active molybdenum cofactor for nitrate reductase and the authors were able to confirm the observation of Pienkos *et al.* (1977) that nitrogenase and nitrate reductase do not share the same molybdenum cofactor.

It will be obvious from this discussion of *Rhizobium* mutants that we still know very little about the basic metabolic properties of rhizobia and the effect of perturbations in metabolism on the symbiotic properties of the bacteria. The techniques for culturing, mutagenizing and genetically manipulating fast-growing rhizobia are now well established. Unfortunately progress with the slow-growing rhizobia has been less rapid, though some metabolic mutants have been isolated. Until fast-growing rhizobia can be routinely induced to fix nitrogen *ex planta* studies of nitrogen fixation by the rhizobia will be restricted to studies of nodules or isolated bacteroids.

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