ISOLATION OF IS ELEMENTS FROM *RHIZOBIUM* STRAINS. CHARACTERISATION OF *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS USING ISOLATED IS ELEMENTS.

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ABSTRACT

The broad host range plasmid pSUP104 carrying the streptomycin sensitivity gene - S12, was mobilized from Escherichia coli S17-1 to Rhizobium meliloti 2011, R.leguminosarum 897 and two Bradyrhizobium japonicum serogroup 123 strains. Transconjugants were purified from the two Rhizobium strains. The plasmid pSUP104-S12 failed to replicate in the B.japonicum serogroup 123 strains and no transconjugants were isolated. The plasmid pSUP104-S12 induced a streptomycin sensitive phenotype in R.meliloti 2011 and R.leguminosarum 897 which were previously streptomycin resistant. The selection for streptomycin resistant revertants carrying the pSUP104-S12 plasmid caused insertion of IS elements directly into the S12 gene. Two IS elements were isolated, one ISRm2 is a 1.25 Kb IS element isolated from R.meliloti 2011, the other ISR11 is a 2.7 Kb IS element isolated fron R.leguminosarum 897.

Both IS elements caused deletions of the pSUP104-S12 plasmid in a Rec A- E.coli strain.

Hybridization analysis using ^{32}P labelled pSUP104-S12::ISRm2 as a probe with EcoR1 digests of total chromosomal DNA of various Rhizobium and B.japonicum serogroup 123 strains revealed that ISRm2 was particular to R.meliloti strains. By the same method ISR11 was found in all the Rhizobium strains tested and in most of the B.japonicum serogroup 123 strains. It would be possible to use ISR11 as a probe in DNA fingerprinting to identify a B.japonicum serogroup 123 inoculum from an indigenous B.japonicum serogroup 123 in

field trials of a serogroup 123 inoculum.

Both IS elements were implicated in DNA rearrangements in *Rhizobium* strains.

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INTRODUCTION

NITROGEN FIXATION

Nitrogen is a key building block of the protein molecule upon which all life is based, and it is thus an indispensible component of plants, animals and micro-organisms. Nitrogen fixation is the term used to describe the conversion of inert atmospheric dinitrogen to ammonia,which is the form in which it is metabolisable by plants and is described by the equation:

$$N_2 + 3H_2O \Longrightarrow 2NH_3 + 3/2O_2$$

The earths atmosphere consists of 79% Nitrogen in its inert form dinitrogen. This abundance of nitrogen in the atmosphere and its relative scarcity on the earth's surface suggests that nitrogen fixation is the rate limiting step. (Figure 1).

Fixation is largely a biological process contributing over 70% of total global nitrogen input (2×10^8 metric tonnes of nitrogen per year). Non-biological fixation by such means as the Haber-Bosch process, electrical storms, ultra-violet radiation and the internal combustion engine supplies only about 25%. The rising costs of producing nitrogenous fertilizer by the Haber-Bosch process (which is dependant on oil or natural gas) and the related transportation costs result in it becoming economically prohibitive for many countries to buy this type of fertilizer. In developed countries there is a need to reduce the input costs to agriculture in order to increase the farmers return without price increase. Their requirements could be met in certain

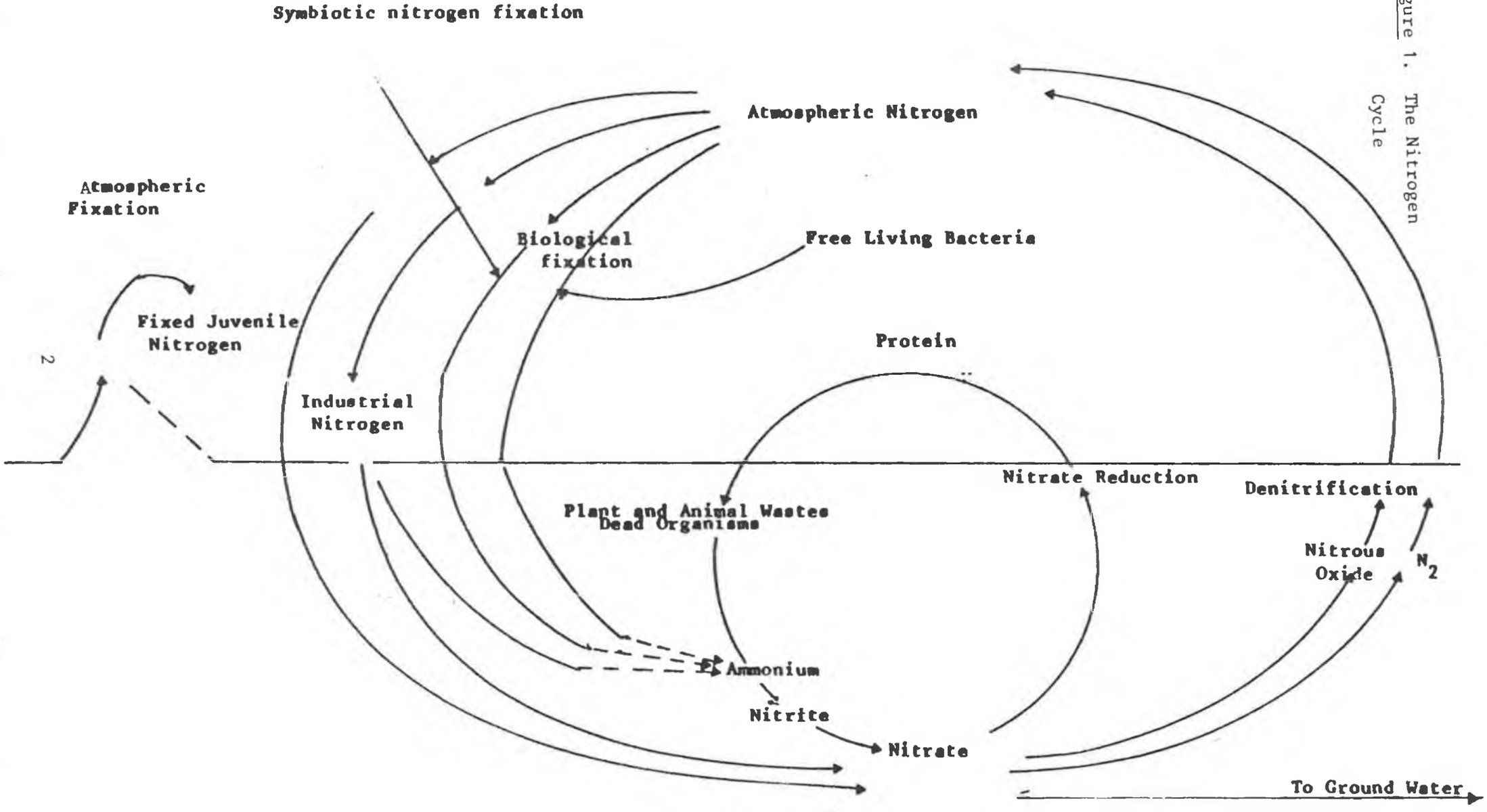


Figure 1. The Nitrogen Cycle

circumstances by the use of inoculant microorganisms capable of fixing nitrogen by symbiotic or asymbiotic means. Trees in tropical forests whose roots are colonised by the actinomycete Frankia are capable of thriving in soils devoid of nitrogen and also in phosphorous deficient soils (Dommergues et al., 1983). Incorporation of the cyanobacterium Azolla into paddy rice soils in the Far East provides sufficient nitrogen for crop growth (Dart, 1983). Cereal crops innoculated in the Middle East with Azospirrilum spp. have been shown to give increased yields. The most widely investigated symbiotic nitrogen fixing bacteria are members of the genus Rhizobium which nodulate legumes. A leguminous crop if inoculated with effective strains of rhizobia can add up to 200kg of nitrogen to the soil per hectare per year. In the United States alone legumes such as alfalfa, soybean and peanuts contribute 2.4 million tons of nitrogen a year to the soil through symbiotic nitrogen fixation (Ahmad and McLaughlin, 1985). Much field and laboratory work to develop competitive and effective inocula and appropriate farming systems needs to be carried out to capitalise on the potential to replace nitrogenous fertilizers with natural inoculum.

RHIZOBIUM TAXONOMY

Rhizobium is a genus of gram negative soil bacteria which are able to form symbiotic relationships with legumes. In specialised structures called nodules they are able to fix nitrogen. Taxonomic groups are based on the nodulated host

RHIZOBIUM SPECIES	GROWTH RATE	PLANT HOSTS
R.meliloti	Fast	Medicago
R.trifoli	Fast	Trifolium
R.leguminosarum	Fast	Pisum, Vicae
R.phaseoli	Fast	Phaseolus
R.fredii	Fast	Glycine
R.lupini	Variable	Lupinus
R.japonicum	Slow	Glycine
R.spp. cowpea.	Slow	Vigna
R.parasponiae	Slow	Parasponiae

The cross inoculation groups are not as exact as shown. Many bacteria nodulate hosts outside their grouping and many fail to nodulate all members of that group. The classification of rhizobia can be broadly divided into fast and slow growing groups (Long,1983) . The slow growers such as members of R.japonicum and R.cowpea groups are alkali producing on agar. They lack an NADP-linked 6-phosphogluconate dehydrogenase (6-PGD E.C.1.1.1.43) the key enzyme of the hexose monophosphate pathway. They use the Entner-Dourdoroff pathway instead (Martinez-De Drets and Arias, 1972). The fast growers which produce acid on agar are members of the R.meliloti, R.leguminosarum. R.trifoli and R.phaseoli groups.

There are exceptions to these groupings. Strains have been isolated which are fast growers yet nodulate Glycine max.(

var. Peking). They form ineffective nodules on most though not all commercial soybean cultivars (Israel et al., 1986, Stanley et al., 1986). Lotus species are nodulated by fast and slow growing strains as are the various tropical legumes such as cowpea which remain largely unstudied.

This inconsistency has led to the analysis of metabolic requirements, serology and DNA homology as an outline for taxonomic groupings. R.trifoli, R.leguminosarum, and R.phaseoli were indistinguishable on the basis of DNA homology, and further studies involving the exchange of plasmids encoding functions for nodulation and nitrogen fixation between these strains have altered their nodulation patterns (Johnston et al., 1979; Hooykaas et al.; 1981). The chromosomal map of the plant pathogen Agrobacterium is co-linear with the known fast growing rhizobia maps especially R.meliloti showing close relatedness of the genera . Agrobacterium containing the symbiotic plasmid of R. meliloti can be made to nodulate Medicago. R. meliloti and R.leguminosarum containing the symbiotic plasmid (pSym) of R.fredii 191 can nodulate soybeans (Appelbaum et al.. 1985). As a result of the major differences in metabolism and DNA homology between the fast growers and the slow growers, their only common bond being that they both nodulate legumes, it has been proposed to group the slow growers into a distinct genus Bradyrhizobium.

The generic name Bradyrhizobium distinguishes the slow

growing strains of rhizobia (the greek Bradus meaning slow) from the fast growing strains retaining the generic name Rhizobium. Two criteria in use for this delineation include moderate turbidity after 3-5days growth an agitated broth, and production of alkaline reaction in mineral salts-mannitol medium after 28 days at 27°C, though acid-alkali production has been shown to have little correlation to doubling time for some cowpea and B. japonicum strains. (Hernandez and Focht, 1984).One alternative is to use the presence or absence of 6-PGD in strains of intermediate doubling time of between 8-11h as a physiological criterion for rhizobia taxonomy.

As a genus Bradyrhizobium remains relatively poorly characterised genetically because of its distinctive slow growth and the inefficiency of classical gene transfer methods. In Rhizobium genes for nodulation and nitrogen fixation are carried on large plasmids known as symbiotic plasmids. (Hooykas et al.. 1981 ; Rosenburg et al., 1981 ; Batut et al.. 1985; Truchet et al., 1985) and though plasmids have been found in some Bradyrhizobium strains no symbiotic plasmids have been detected(Masterson et al., 1982 ; Broughton et al., 1983). The structural nitrogenase genes (nif) genes have been isolated from R.japonicum gene libraries using cloned Klebsiella nif genes(Hennecke , 1981). In contrast to the single nifHDK operon of K.pneumoniae and Rhizobium ,two separate transcriptional units were found. One, nifH codes for the nitrogenase Fe

protein with its own promoter and the other nifDK codes for the nitrogenase Mo-Fe protein. (Fuhrmann and Hennecke, 1982 ;Hennecke et al., 1983; Kaluza et al., 1983 ; Fischer and Hennecke, 1984). On the basis of amino acid sequence homology of the nifH gene product B.japonicum species have been placed equidistant in evolutinary terms from the fast growing rhizobia and the gram negative non- symbiotic azotrophs (Furhmann and Hennecke, 1984). Also fixABC genes which are located in one operon in R. meliloti have been found to be in two transcriptional units,fixA and fixBC in B. japonicum (Furhmann, Fischer and Hennecke, 1985 ; Noti et al., 1986 ; Earl et al.. 1987). This has given more credence to their classification as a separate genus (Furhmann and Hennecke, 1984).

When numerous B. japonicum strains were isolated from different geographical locations and hybridised to nifDK and presumptive nod genes, two distinct evolutionary types were seen and a marked difference was observed between these strains and the fast growing isolates from nodules of Glycine max (var Peking).(Stanley, Brown and Verma, 1985). This genomic difference could be seen at the phenotypic level. One group had nitrogenase activity ex planta on gluconate-glutamate media while the other group had not (Huber, Argual and Keisler, 1984). Differences in exopolysaccharide produced by these two evolutionary lines was also found.

SOIL INOCULATION STUDIES WITH RHIZOBIA

Much research work has been carried out investigating the nitrogenase, nodulation and nitrogen fixation genes in rhizobia. This work alone will not lead to the production of a useful inoculum. Many factors are involved, such as competition and persistance in the soil. Nif- mutants of B.japonicum retain the same competitiveness as wild type strains(Hahh and Studer, 1986). B.japonicum can be taken as an example to show the problems encountered in finding the ideal inoculum. Soybeans are a major crop in the Mid-Western States in the U.S.A.. They are nodulated by B.japonicum and the serotype which predominates is serogroup 123 which occupies up to 60-100% of the nodules on host cultivars (Damargi et al., 1967). Inoculation of soybeans with selected strains of B.japonicum has been notably unsuccessful in influencing nodulation or enhancing nitrogen fixation in regions where soybeans have been cultivated previously (Ham et al., 1971 ; Moawad et al., 1984 ; Ellis and Schmidt, 1987). To form an understanding of the basis of competition, relationships between three serogroups 110, (which has a higher nitrogenase activity in laboratory plant tests than serogroup 123) 123, and 138 have been examined over two growing seasons with several hosts, with and without inoculation. The three competitors were enumerated in rhizosphere and non rhizosphere soil by immunoflouresence with serogroup specific antibody (a widely used technique which will be discussed in a later paragraph) . In fallow

soil indigenous Bradyrhizobium ranged from 4 x 10^5/g of soil in Autumn to 1 x 10^4/g in Spring with serogroup 123 varying from 10-50% of the total respectively. In the rhizosphere the indigenous population increased gradually in number during the first few weeks after planting with no sign of dominance by serogroup 123, though it dominated nodule composition. B.japonicum populations were greater in host than non-host rhizosphere, but in both they were greatly outnumbered by other bacteria. The success of serogroup 123 in achieving nodulation does not appear to be by superior colonisation of host rhizosphere. This predominance of serogroup 123 existed even where serogroup 110 had been inoculated at levels of 10^7 per seed. Quantitative studies in greenhouse conditions found that the ratio of nodules formed by the applied inoculant to the indigenous soil strains was related to the ratio of Rhizobium cells in inoculum to the cells in the soil (Amarger and Lobreau,1982). Difficulties arise in correlating results from greenhouse trials to the field situation as competition in growth pouches does not mimic the natural environment.

The growth phase of the inoculum may have a bearing on competition with reference to initiation of infection. Research has shown that log phase cultures of serogroup 110 and 138 have greater nodulation potential than stationary phase cultures. This effect is minimal in serogroup 123 (Bhuvaneswari et al.,1983).

Colony variants of pure cultures have been investigated, including some producing large mucoid mannitol utilising

colonies which are ineffective symbiotically and others yielding small dry colonies non-mannitol utilising which are effective (Kykendall and Elkan, 1976).Data show a link between the production of a symbiotically effective inoculum and four factors: increased salt sensitivity, higher non-associative nitrogen fixation potential , high symbiotic nitrogen fixing potential and small non-slimy colony morphology (Upchurch and Elkan, 1977). These colony variants arise spontanously from pure cultures. The discovery of mannitol utilising colonies with slimy morphology which have nitrogen fixing potentials equal to or greater than their non-mannitol using dry colony type parents has cast doubt on earlier assumptions (Kuykendall and Elkan, 1986; Mathis et al., 1986 ;Mathis et al., 1986)

The host exerts a great effect on inoculum μ a particular soybean genotype may be nodulated more efficiently by a certain serogroup of Bradyrhizobium (Kvein et al., 1981). Pre-treatment of an inoculum with soybean lectin has been shown to affect nodulation showing further the effect of the host on the innoculum (Halverson and Stacey, 1986).

The presence or absence of a hydrogenase uptake system (HUP) in rhizobia has significance in agriculture in terms of economic benefit. During nitrogen fixation 25-30% of the reducing power is used for the production of hydrogen gas which can be a wasteful product. The strains that possess an uptake hydrogenase system (HUP+) can recycle the hydrogen for the synthesis of ATP. Thus HUP+ rhizobia are more

efficient in their energy metabolism than HUP- strains (Scot et al.,1986). They had been thought to produce up to 50% more plant dry matter than HUP - strains, though a recent report has contradicted these results (Drevon et al.,1987 ; Saini et al., 1987). The oxidation of hydrogen in Hup + strains helps reduce the intracellular oxygen concentrations, thus protecting the oxygen sensitive nitrogenase enzyme. Inoculant industries are supporting the idea that HUP- rhizobia should be genetically altered to HUP+ strains for inoculum production (Lambert et al.,1987). Many of the Rhizobium spp. screened have been shown to be HUP - but 25% of B.japonicum examined were HUP+, although variation occured even within serogroups (Keyser, Weber and Uratsu, 1984). The majority of cowpea rhizobia have been shown to be HUP+ (Ahmad and Mclaughlin, 1985).

Inoculation is not simply a matter of providing competitive, effective B.japonicum for soybeans in soils where none are present. The inoculum must provide superior strains, which are able to establish themselves in soybean nodules in soils containing naturalised strains of B.japonicum. Increased soybean yields from the use of inoculum have only been shown in soils free of B.japonicum. Much of the difficulty in research on the effect of an inoculum arises from the problem of identifying an inoculum strain against the indigenous rhizobia which may be of the same serogroup. Many methods are currently used.

SEROLOGY: Recognition of strains by serology is on the basis of the antigenic properties of bacterial cells. Animals injected with an antigen produce antibodies which are a group of related globulin proteins that are capable of specific non-covalent binding to antigen molecules. These antibodies can be harvested from the serum and used for serological tests. Antigens which induce this immune response can be either proteins or polysaccharides. In addition many types of molecules which are not intrinsically antigenic become antigenic when attached to proteins or polysaccharides. These molecules include lipids, nucleic acids, carbohydrates and other small molecules (Stanier et al., 1977). Surface antigens of rhizobia may be shared among strains within a species and some are common to certain strains outside the present specific boundaries. Cross reactions occured between the R.leguminosarum. R.trifoli. R.phaseoli groups. R.meliloti was distinctive, though similarities between it and Agrobacterium spp. were found (Vincent, 1981).Cross reactions have also been noted among the slow growing rhizobial groups. The somatic or O-antigens, which are the polysaccharide components of the lipopolysaccharides in the outer layer of the cell envelope, are the most widely used antigens for the production of antibodies. They are more strain specific than the other surface antigens such as the K - antigen (the capsular polysaccharides), or the H - antigens (the flagellar proteins).The internal antigens such as enzymes seem to be

widely distributed within a genus and are therefore relatively non specific (Vincent, 1970).

Three types of antigen - antibody reactions are commonly used. The immune diffusion reaction occurs between soluble antigens and antibodies. It is recognised by bands of precipitate forming in agar where different diffusing antibodies and antigens meet at optimal concentrations. It permits antigenic identity and/or similarity to be assessed. The agglutination reaction occurs between particulate antigen and antibodies and is recognised by the clumping of cells when a positive reaction has occured. The fluorescent antibody reaction occurs between particulate antigens and antibodies conjugated with a fluorescent dye such as fluorescein isothiocyanate and is recognised by the use of a microscope equipped with an ultra-violet light source and special filter (Weaver and Frederick, 1982). In ELISA test the antigen is adsorbed onto a solid surface (usually the wells of a microtitre plate). An enzyme conjugated antibody is then added. Binding is assessed by adding substrate specific for the enzyme conjugated to the antibody.

The term πserotypeπ is used to designate a pure culture which reacts with a certain antiserum. The term rhizobial πserogroupπ refers to any isolate or nodule suspension which reacts to a certain antiserum. Pure cultures from the same serogroup may differ in other characteristics making it possible to have several serotypes of rhizobia in the same serogroup. B.japonicum serogroup 123 shares common antigens with other B.japonicum serogroups such as serogroup 127 and

129. To eliminate cross reaction such as this, the antiserum for all methods must be reacted with the heterologous strains to remove common antibodies (Schmidt et al., 1986).

PHAGE SUSCEPTIBILITY: According to the degree of specificity between the phage and its host, resistance or suceptibility to lysis by a particular phage may be used as a means of strain identification. If the phage possesses an adsorbtion site that is chemically complimentary to a specific receptor site on the bacterial cell surface - irreversible adsorbtion occurs. The receptor sites can be found in the lipoprotein outer layer of the cell wall, in the lipopolysaccharide layer or on cell appendages such as pili and flagella (Stanier et al., 1977). The plaque method can be used to isolate from the rhizosphere phages that are lytic for a particular strain or species of rhizobia. Soil from the rhizosphere of a leguminous crop can be taken and shaken in water. The supernatant is the filter sterilised and stored over chloroform. Aliqouts are mixed with the rhizobial strain and plated on agar. Any zones of clearing or plaques that appear represent phages lytic for that strain. The phage can be harvested and used to identify strains that are susceptible to it (Vincent, 1970). Phages can also be cross reactive between serogroups such as the phages isolated for B.japonicum serogroup 123 (Schmidt et al. 1986). Phages have been isolated that distinguish between nodulating and non-nodulating mutants. A difference in the cell surface of the

mutants accounting for the failure to nodulate is thought to be the reason for the non-adsorbtion of the phage (Stacey et al., 1984). Positive selection of nodulation deficient mutants of R.phaseoli by phage has been used (Raleigh and Signer, 1982)

SUSCEPTIBILITY TO ANTIBIOTICS : Antibiotic resistance is a very useful marker in ecological, field and laboratory studies. Antibiotics are relatively easy and inexpensive to obtain in comparison with the development and purchase of antiserum. They overcome some problems of serological methods such as the inoculum belonging to the same serogroup as the indigenous rhizobia. Incorporation of the appropriate concentrations of a particular antibiotic permits differential counts of appropriately labelled strains or the quantitative determination of strains occupying nodules (Lieberman et al. 1986). Strains of rhizobia differ in their natural resistance to various antibiotics. Josey et al. (1979) screened several strains of R.leguminosarum and R.phaseoli against various concentrations of eight different antibiotics and found unique patterns of intrinsic resistance. The patterns of resistance or susceptibility were used for the reliable identification of nodule isolates from inoculated host plants. The use of intrinsic antibiotic resistance avoided the possibility of reduced competitiveness and symbiotic effectiveness caused by mutation to resistance to high levels of antibiotics (Kremer and Peterson,1982 ; Turco et al.,1986). The spontaneous mutation of a strain may give

rise to secondary mutations,for example, mutants growing in the presence of neomycin and viomycin are frequently ineffective . Cyclohexamide is incorporated into selective antibiotic plates to inhibit fungi which would contaminate the test. Identification of bacteria in small nodules is more readily achieved using antibiotic resistance than serology (Weaver and Frederick, 1982).

METABOLIC AND GENETIC MARKERS:

Other metabolic and genetic markers that differentiate between strains are not widely used for identification purposes but may be developed. Auxotrophs or mutants in metabolic pathways may be ineffective or competition deficient because they are in a stressed condition. One metabolic trait that is linked to nitrogenase activity is hydrodgen uptake (HUP) . Monitoring root systems axienically and in soil for H_2 evolved as a means of assessing rhizobial competitiveness in conjunction with antibiotic resistance showed that this method overestimated the competitiveness of HUP+ strains while underestimating the competitiveness of HUP- strains. Hydrogen oxidising bacteria which colonise the rhizosphere can affect the results and until their contribution to the hydrogen evolution of a plant is assessed the HUP method con only be used to provide circumstancial evidence of competitive success (El Hassan et al.. 1986).

Polyacrylamide gel electrophoresis of soluble protein shows heterogeneity in the profiles of various strains of

Agrobacterium which could be used for taxonomic studies (Alarcon et al., 1987). In rhizobia, variation in three enzymes has been examined. These are glucose-6-phosphate dehydrogenase, tetrazolium oxidase and beta-galactosidase (Young, 1983; Young et al., 1987).

DNA FINGERPRINTING: The analysis of the indigenous plasmid profile of a rhizobial strain by gel electrophoresis may help with its identification. Rhizobium spp. contain large plasmids, some encoding symbiotic functions. This is not the case with Bradyrhizobium. The restriction analysis of total DNA after electrophoresis in a 1% agarose gel gives a characteristic pattern for a strain though these patterns are very similar within a species and therefore identification may be difficult. What is needed is a probe which will conclusively identify one strain from another and possibly elucidate evolutionary relationships between strains. Many hybridization probes have been used, such as the nif region. Kalusa et al. (1986) speculated that IS elements isolated from a genus could be used as a hybridization probe for the identification of an isolate. Repeated sequences isolated from R.trifoli could also be used for strain identification (Watson and Schofield, 1985).

THE GENETIC MANIPULATION OF RHIZOBIA

To facilitate the analysis and manipulation of the rhizobium-legume symbiosis, vector systems have been developed which facilitate efficient transposon mutagenisis, site specific insertion of selective markers, complementation studies, mobilization of cryptic plasmids

and maintanence of a vector in rhizobia. Two types of vector have been constructed, one has a narrow host range, the other a broad host range. Both vectors are mobilizable into rhizobia, which is necessary as no reproducable transformation system has been found.

NARROW HOST RANGE VECTORS: These vectors consist of the mob site of the broad host range plasmid RP4, inserted into commonly used Escherichia coliplasmids. They were constructed by the partial digestion of RP4 by Sau3A and the linearisation of the E.coli vector with BamHI. After ligation and transformation the resulting plasmids were screened for their ability to be mobilized. The resulting plasmids contain the OriT region that enables the vector to be transferred in the presence of RP4. This OriT region (mob site) is recognised in trans by the transfer functions of the conjugative Inc P plasmids . These vectors replicate only in E.coli but rhizobia can act as recipients in conjugal transfer , as they do for RP4 (Simon et al., 1983 ; Simon et al., 1985). Failure to replicate in rhizobia has made them very convenient for use in random or site specific transposon mutagenisis. They overcome the problems encountered in previous systems where the suicide vector overcomes its mutation and is stably maintained in the conjugal recipient though integration of the vector can occur (Meade et al., 1982 ; Simon et al., 1985). Transposon mutagenisis of cryptic Rhizobium plasmids by Tn 5, containing a cloned RP4 mob site facilitates their

mobilization into other Rhizobium spp. and Agrobacterium.

BROAD HOST RANGE VECTORS: These vectors are derived from the broad host range plasmids RSF1010, a member of the Inc Q incompatibility group or RK2, similar if not identical to RP4 (Barth, 1979). These vectors are non-self transmissable but mobilazable by the Inc P plasmids and are able to replicate in rhizobia. The RSF1010 derivatives combine its useful broad host range properties with the antibiotic resistances and cloning sites of E.coli vectors.Many workers have constructed RSF1010 derivatives (Frey et al.,1985), and some of the most important vectors for the manipulation of rhizobia are derived from pKT210 an 11.8kb chloramphenicol and streptomycin resistant derivative of RSF1010 (Bagdasarian et al., 1979). These vectors were constructed by the partial digestion of PKT210 by Sau3A and its subsequent ligation to an E.coli vector which had been linearised by digestion with low concentrations of Sau3A. After transformation into a mobilizing strain , the transformed bacteria were conjugated en masse with R.meliloti. The resulting transconjugants selecting for one of the vector encoded resistances were the derivatives of the vector with the broad host range and replication functions of RSF1010. These vectors have a high copy number, low molecular weight and numerous cloning sites. They are mobilizable to Rhizobium and Agrobacterium species in which they replicate (Simon et al. 1983; Priefer et al., 1985). Though RSF1010 has a broad host range, the insertion of foreign DNA into non-essential regions of the vector can

cause instability in Pseudomonas putida (Meyer et al, 1982). The Inc Q origin of replication is thought to be unable to function or does not function at a sufficiently high level to allow replication in Bradyrhizobium. The Inc Q plasmids can exist as a cointegrate with RP4 in B.japonicum. Maintenance is likely to be dependant on the RP4 replication functions (Donnelly et al., 1987).

Ditta et al. (1980) constructed pRK290, a derivative of RK2. RK2 could be used directly as a cloning vector, but the lack of restriction sites, thought to be an adaption to broad host range and its large size are a drawback to its routine use. Three widely separated regions of RK2 designated oriV - the origin of replication, trfA and trfB - the regions encoding trans acting replication functions, are neccessary for RK2 replication. A fourth region termed rlx, which encodes a cis-acting function necessary for conjugal mobilization to occur is thought to represent the origin of conjugal transfer (Thomas et al.,1979). pRK290, which contains all these regions and a tetracycline resistance marker, was constructed by the successive restriction af RK2 with various enzymes to eliminate non-essential regions. The resulting plasmid is 20kb in size, with two cloning sites, neither of which have insertional inactivation of the antibiotic resistance gene. pRK290 is non-self mobilisable but can be transferred by a mobilising strain at a high frequency to Rhizobium and Bradyrhizobium, where it is stably maintained. The relatively large size of pRK290 and the necessity to alkaline phosphatase treat the vector to

minimise the overwhelming background of molecules without inserts, which would arise as a result of recirculization of the plasmid, impinge on the successful use of pRK290 as a vector (Ditta et al., 1980).

Both classes of vector described above are mobilizable by RP4, a plasmid of the Inc P incompatibility grouping which is self transmissible and stably maintained in all Gram negative bacteria studied. To avoid the undesirable co-transfer of RP4 with the vectors, mobilising strains have been constructed which have derivatives af RP4 integrated into the chromosome. The transfer functions of RP4 are used to build conjugation bridges to recipient strains and mobilise the vector. These strains were constructed by forcing two different derivatives of RP4 to co-exist in a Rec A deficient E.coli. The incompatibility of the two plasmids forces one of them into the chromosome. The transfer genes of the integrated plasmid are still expressed and can be used to transfer plasmids containing the RP4 mob site. The antibiotic resistance markers of the integrated RP4 have been inactivated by insertion or deletion. The transfer frequency of the mobilizable vector is as high as that of the wild type RP4 (Simon et al., 1983).

PROKARYOTIC MOBILE GENETIC ELEMENTS

Controlling elements in maize, discovered by McClintock in the 1940's were the first mobile genetic elements to be described with precision. Since then mobile genetic elements

have been observed throughout both the prokaryotic and eukaryotic kingdoms. Prokaryotic mobile elements defined as genetic entities capable of inserting as discrete, non-permuted DNA segments in the genome can be grouped into three classes.

Class I : Insertion sequence (IS) like elements.

Normally ranging from 750-1500 base pairs (bp) they contain only genes relevant to their own transposition. They are compactly organised containing one or more structural genes, regulatory information and transposition determinants, sometimes overlapping, all within a very limited coding capacity. Transposition intermediates and the mode of action of the gene products of the IS elements remain largely unknown. Composite elements such as Tn5 and Tn10, are flanked at the ends by IS elements. These IS elements, which are capable of independant transposition, are required for transposition of composite elements which are therefore grouped as Class I elements (Kleckner, 1981; Iada et al., 1983).

Class II: Tn3 and related elements.

They contain a phenotypic marker such as antibiotic resistance. Transposons as they are generally termed are self regulatory, with transposition activity coupled to expression of phenotype. Transposition is by a two step mechanism. The first step is the fusion of the donor and recipient DNA molecules by the duplication of the transposon. In the second step, the duplicated copies undergo recombination to yield precise transposition, unlike

composite elements where cointegrate formation can be stable. Tn3 encodes three functionsμ beta-lactamase, giving ampicillin resistance and two proteins TnpA and TnpR. TnpA promotes cointegrate formation and TnpB promotes cointegrate reduction (Kleckner, 1981 ; Heffron, 1983).

Class III: Transposing bacteriophages Mu and D108.

Both phages are capable of lytic or lysogenic growth. The two phages show 95% DNA sequence homology, similar functional organisation and partial functional cross reaction. Two Mu genes A and B, located adjacent to one another, close to one end of the phage genome, are required for Mu mediated transposition. Though homologies between the ends of Mu exist they are not inverted repeats. A 7-bp sequence which exists once at one end and five times at the other, occurs near the ends of several class II elements suggesting a possible recognition site by a host protein or some other mechanistic relationship (Kleckner, 1981).

Class I and II elements share several features. Transposition of an element involves duplication of the element alone, precise cleavage at the end of an element and its ligation to the target DNA which is cut in a specific way. Transposition is accompanied by the duplication of the target sequence, though this does not occur with IS91 (Diaz-Aroca et al., 1987). The length of the duplication is characteristic of a particular element. IS like elements generate typically 9bp, repeats but this can vary from 3-12bp. The Tn3 family generate 5bp repeats. The repeats

probably result from the target molecule being cleaved at staggered positions in the two DNA strands, subsequent ligation and replication. The ends of transposons (Class I and II elements) are themselves short 9-40bp inverted repeats. Mutations in the inverted repeats are non-complementable and affect transposition activity. Insertion of a transposon in the genome can give rise to polar mutations. Some elements carry a site for the transcription termination factor rho causing premature termination of transcription (Das et al., 1977). Gene turn-on can also be activated as a result of adjacent insertion. This "turn-on" effect is dependant on the orientation of the transposon and can be caused by the internal rearrangement of sequences in the transposon or because the ends of some transposons containing promoter like sequences (Glansdorff et al., 1981; Scordilis et al..1987).

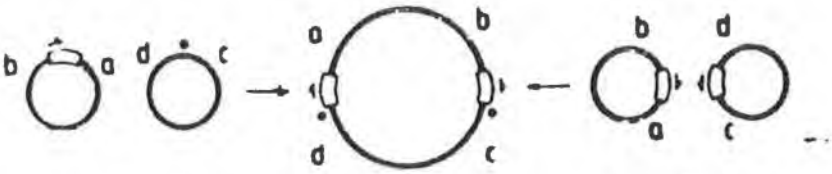
The ability of transposons to promote DNA rearrangements even in Rec - strains is of great evolutionary significance. All transposon promoted events can be accounted for by six type of events shown in Figure 2(i-vi). Transposition and cointegrate replicon fusion events (i + iii) are intermolecular. Duplicate inversion and deletion are intramolecular (iv + v). The adjacent insertion of a second element without altering the intervening material can also occur. Inverse transposition is characteristic of composite elements (Munster and Shapiro, 1981; Saedler et al., 1981). Mutations and DNA re-arrangements, inexplicable by conventional techniques, have been the means of discovery

Figure 2. DNA rearrangements caused by transposable genetic elements.

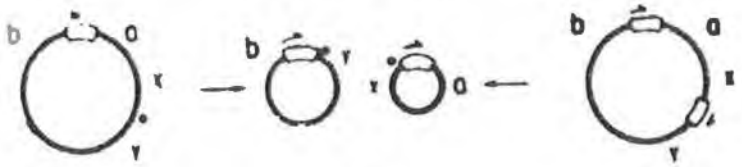
(i) Transposition

(ii) Excision

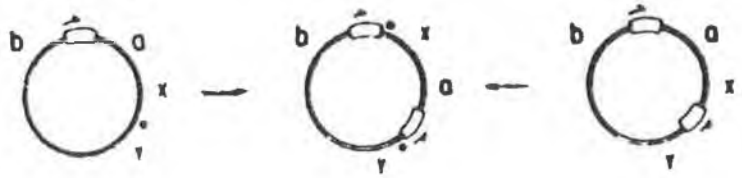
(iii) Cointegration



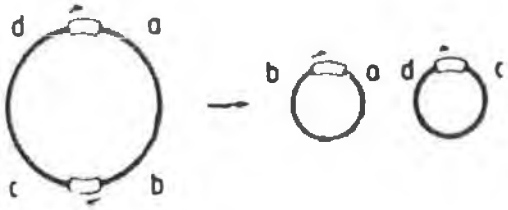
(iv) Deletion



(v) Inversion



(vi) Resolution



of many transposons. Researchers interested in the regulation of gene expression of various E.coli operons discovered pleotrophic mutations in structural genes for pathway enzymes. These mutations were caused by the insertion of 700-1500bp segments of DNA. They exerted strong polar effects on expression of other genes distal to the operon promoter (Saedler, 1977). In electron microscope heteroduplex analysis, these insertion sequences, now known as IS1,IS2,IS3 and IS4, appeared as inversion loops with duplex stems in a molecule formed between inverted repeats on a single strand. Alternitavely they appeared as out-of-register structures formed between direct repeat sequences in two single strands from the genome. These IS elements have been located in the gal, lac and arg operons in E.coli (Reif and Saedler, 1977 ; Ammed et al.. 1981). A novel technique involving the denaturing of intact plasmid DNA, followed by a rapid 30 second renaturing of any inverted repeat sequences present and the subsequent digestion of single stranded DNA by S1 nuclease, has aided the detection of IS elements . Duplex DNA is resolved by electrophoresis through a 1.4% agarose gel. Inverted repeats of transposons have been successfully isolated by this method from plasmid and chromosomal DNA (Ohtsubo and Ohtsubo, 1977).

The R plasmids of the R (fi+) group can dissociate into two autonomously replicating units. One unit is the resistance transfer factor which codes all the functions neccessary for cell to cell contact, allowing the transfer of the plasmid.

The drug resistance determinate, carrying the antibiotic resistance genes being the other. The two regions are separated by two copies of IS 1 in the same orientation. The presence of IS1 can give rise to plasmid rearrangement, leading to amplification of antibiotic resistance (Saedler, 1977 ; Chandler et al., 1981). A tetracycline resistance region bracketed by two IS 3 elements was isolated. The wide spread resistance to antibiotics and the evolution of R plasmids may be accounted for in part by the existance of class I and II elements (Heffron et al., 1977; Mitsuhashi et al., 1977 ; Iada et al., 1981).

Since the discovery of IS elements in E.coli, numerous other IS like elements have been revealed throughout the prokaryotic kingdom. Corynebacterium diptheria. is a gram positive bacterium, which after lysogenisation by a temperate bacteriophage carrying the toxin (tox) gene, acquires the ability to produce diptheria toxin. A 1.5Kb insertion sequence was discovered in C.diptheria belafonti 1060 which had between fifteen and twenty five copies in the genome. Epidemological studies on Corynebacterium strains, using the IS element, found that the element was present in most strains of C.diptheria. Isolates from the same region had similar hybridization patterns and interstrain relationships could be established (Rappuoli et al., 1987).

A 1.4Kb IS element ISRm1 which transposes preferentially into the nif region has been isolated from R.meliloti (Ruvkun, 1980 ; Ruvkunet al., 1982). After Tn5 mutagenesis 33 symbiotic mutants from 6,000 clones were isolated which

failed to show Tn5 insertion. Though there are about ten copies of ISRm1 in the R.meliloti genome πhot spotsπ for insertion appear to be into three EcoR1 fragments which map in the nif region. Given that ISRm1 transposes into the nif region where it causes fix - mutations, it is still not thought to be a controlling mechanism . No copies of IS Rm1 were found in other rhizobia even in other strains of R.meliloti.

RP4 was found to be unstable in R.lupini and examination of these RP4 mutants showed a 1.15 Kb insertion which was thought to be responsible for the instability of RP4 (Burkhardt et al., 1979; Priefer et al., 1981). ISR1 has one restriction site for the enzymes HindIII, PstI and BamHI. ISR1 can insert into three regions in the RP4 plasmid, giving rise to mutant phenotypes. Ampicillin and kanomycin sensitivity and tra- phenotypes arise by insertion and subsequent deletion. ISRI inserts within Tn1 without affecting the expression of beta lactamase. Tn1::ISR1 is found to transpose at a 15-20 fold higher rate than wild type Tn1.

Two repeated sequences similar to IS elements have been isolated from B.japonicum . They are clustered around the nif region but have not been found to influence nif or nod related functions. RSRja is 1.126bp long with one HindIII and ClaI site and is repeated twelve times in the genome. RSRjb is approximately 950bp long and is repeated at least six times (Kaluza et al., 1986). Both repeated sequences

were isolated from a cloned 35Kb region from the B.japonicum genome containing the nitrogenase genes. Probes from the 5' flanking side of nifD hybridised to numerous restriction fragments. Sequencing of the ends of RSRja has shown them to be inverted repeats. It is thought possible that these repeated sequences may be involved in deletion in the genome but this has not been conclusively proven (Regensburger et al., 1986). These IS elements may prove useful for strain identification.

STREPTOMYCIN SENSITIVITY V. RESISTANCE.- THE S12 GENE

Exposure of sensitive cells to Streptomycin causes a variety of metabolic effects, including the inhibition of protein synthesis, stimulation of RNA synthesis, inhibition of cellular respiration, membrane damage and cell death. Streptomycin binds to the ribosome and causes inhibition of protein synthesis by the accumulation of abherrent initiation complexes. Three phenotypic responses to streptomycin, resistance, dependance or sensitivity are determined by a single ribosomal protein - S12. A single step mutation in the strA gene encoding ribosomal protein S12 can lead to a high level of streptomycin resistance. The resistant ribosome may fail to bind to the drug or change in conformation so that the effect of the drug if bound is modified. In Merediploid strains of E.coli heterozygous for the strA locus , sensitivity to streptomycin predominates over resistance. The dominance of sensitivity over resistance can be explained by the fact that up to 90% of

cellular ribosomes may be bound to mRNA as polysomes at any given moment. Therefore most or all mRNA molecules might be blockaded by inhibited, sensitive ribosomes in the presence of streptomycin. The formation of stable initiation complexes would prevent protein synthesis. After the formation of complexes by all the sensitive ribosomes protein synthesis might be resumed by the drug-resistant ribosomes. This would be self correcting due to the synthesis of new sensitive ribosomes (Gale et al., 1972).

The broad host range high copy number plasmid pSP104 containing a cloned 850 bp sequence - the 'S12' gene was kindly donated by R. Simon, Bielefeld University. The S12 gene in pSUP104 confers streptomycin sensitivity on streptomycin resistant cells. the aim of this project was to introduce the 'S12' gene into streptomucin resistant Rhizobium and Bradyrhizobium strains, which would then acquire a streptomycin sensitive phenotype. It was proposed that the selection for resistance to streptomycin by the inactivation of the S12 gene would lead to the isolation of indigenous IS elements. Isolated IS elements could then be used for strain identification, investigation of evolutionary relationships and examination of the function of IS elements in genomic rearrangements in Rhizobaciae.

MATERIALS AND METHODS

M. 1. BACTERIAL STRAINS AND PLASMIDS.

STRAIN		Phenotype/Genotype/ Additional Information	Source or reference
E.coli	C600	F-, *thi*, *thr*, *leu*, *lac* *sup*E44,	M. O'Connell
" "	S17-1	RP4-2[Tc::Mu][Km::Tn7] Tp-Sm *pro*, *res*-, *mod*+,	Simon *et al*., [1983]
""	CSH52	*ara*, *pro*,*str*A	M.O'Connell
" "	294	*pro*-, *rec*+, *res*-, *mod*+	M.O'Connell
R.meliloti	2011	Nod+ Fix+ Str^r	M.O'Connell
"	2011rif^r	Spontaneous mutant of strain 2011	This study
"	65	Wild Type	M. O'Connell
"	65c	Spontaneously cured of a cryptic plasmid	"
A.tumefaciens	1060	Derivativeof *A.tumefaciens* Harbours pRi1855	P.J. Hooykaas Leiden
R.phaseoli	8002	Nod+ Fix+	Lamb *et al*,1982
R.leguminosarum	897	Nod+Fix+*phe-trp*-str^r	Johnston & Beringer,(1976)
"	897rif^r	Spontaneous mutant of 897	This study
"	6015 pSym G1008	Nod+ Fix+ [Trifolium repens var Huai]	M.O'Connell [1987]
"	3855	128 C53 Sm, contains pRL6JI	Brewin *et al*., [1982]
"	B151	128 C53 Cm, cured of pRL6JI	"
"	B164	128 C53 Sm, contains pIJ1008	Gronger *et al*., [1987]

cont'd...

R.trifoli	1027	Nod+ Fix+ Mineral Soil Isolate	M.O'Connell
"	1067	Nod+ Fix+ Mineral soil isolate	"
"	1019	Nod+ Fix+ Mineral Soil Isolate	"
"	5001	Nod+ Fix+	P.J. Hookyaas
B.japonicum	123 ST.P	Nod+ Fix+ *str*^{r} Isolate from St. Paul soil	T. McLoughlin
"	S.D.	Nod+ Fix+ *str*^{r} Isolate from South Dakota soil	"
"	Mn	Nod+ Fix+ Isolate from Minnesota soil	"
"	Lamb	Nod+ Fix+ Isolate from Lamberton, Minn. soil	"
"	ArlS1	Nod+ Fix+ Isolate from Arlington soil	"
"	U	Nod+ Fix+ Isolate from Upper Arlington	"
"	St.P.	rif^r Spontaneous mutant of St.P.	This Study
"	S.D.	rif^r Spontaneous mutant Of S.D.	This Study
"	AR4	Nod+Fix+ Isolate from Wisconsin soil	Isolated for the purpose of this study
"	AR6	""	"
"	AR9	""	"
"	AR11	""	"
"	AR13	""	"

cont'd...

B.japonicum 123	AR14	Nod+ Fix+ Isolate from Wisconsin soil	Isolated for the purpose of this study
"	AR22	"	"
"	AR34	"	"
"	AR41	"	"

Plasmid	Markers	Source or reference
pRK290	Tet^r	Ditta *et al.*,[1980]
pSUP104-S12	Tet^r	R. Simon
pSUP202	Amp^r Cm^r Tc^r	Simon *et al.*, [1983]
pSUP202::Tc^s	Amp^r Cm^r	This study
pSUP202-S12	Amp^r	"
pSUP104-S12:ISRm2	Tet^r	"
pSUP104-S12:ISRl1	Tet^r	"
RP4 Tc	Amp^r Kan^r	M. O'Connell

M. 2 MEDIA

The following media were solidified when necessary with 1.5% Oxoid No. 3 agar unless otherwise stated.

All the chemicals used were analar grade.

Tryptone and Yeast Extract were by Oxoid. Other chemicals by Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Distilled water was used in all preparations. Sterility was achieved by autoclaving at 15lb./in.^{2} for 15 min.

(1) TY medium (TYA/TYB) (Beringer,1974)

This medium was used for the routine culturing of fast growing Rhizobium.

Tryptone	5.0 g
Yeast extract	3.0 g
$CaCl_2 .2H_2O$	0.7 g
H_2O	1 litre
pH	7.2

(2) YM medium (YMB/YMB) (Nuti et al., 1977)

This medium was used for the long term storage of Rhizobium strains and for matings of Bradyrhizobium.

K_2HPO_3	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.4 g
Mannitol	10 g
H_2O	1 litre
pH	6.9

(3) AG medium (AGA/AGB) (Cole and Elkan, 1973)

This medium was used for routine culturing of B.japonicum strains.

$FeCl_3.6H_2O$	0.004 g
$MgSO_4.7H_2O$	0.18 g
$CaCl_2.2H_2O$	0.013 g
Na_2SO_4	0.25 g
NH_4Cl_3	0.32 g
Na_2HPO_4	0.125 g
Arabinose	1 g
Sodium gluconate	1 g
Yeast Extract	1 g
H_2O	1 litre
pH	7.0

M. 3. BUFFERS AND SOLUTIONS

TE Buffer

Tris-HCl	10 mM
Na_2-EDTA	1 mM
pH	8.0

TES Buffer

Tris-Hcl	10 mM
Na_2-EDTA	1 mM
NaCl	50 mM
pH	8.0

For gel electrophoresis:

Tris borate buffer (10 x concentrate)

Tris-HCl	108 g
Na_2-EDTA	9.3 g
Boric Acid	55.0g
H_2O	1 litre
pH	8.3

Tris acetate buffer

Tris-HCl	40 mM
Na-acetate	10 mM
Na_2-EDTA	1 mM
pH	7.8

For Southern Blotting:

Denaturing solution

NaCl	1.5 M
NaOH	0.5 M

Neutralization buffer

Tris-HCl (pH 8.0)	1.0 M
NaCl	1.5 M

SSC (20 X Concentrate)

NaCl	3.0 M
Na-citrate	0.3 M

For Nick Translation:

Nick translation buffer (10 X concentrate)

Tris-HCl (pH 7.0)	0.5 M
$MgCl_2$	0.1 M
DTT	10 mM

DNAase 1 was diluted in nick translation buffer with 50% glycerol and stored at -20°C

For hybridization:

Denhardt's Solution

Serumalbumin	0.1 %
Polyvinylpyrolidon	0.1 %
Ficoll	0.1 %

For autoradiography:

Developer

Kodak LX24	
Developer	100 ml
H_2O	600 ml

Fixing bath

Kodak FX40	
Fixer	200 ml
H_2O	800 Ml

For DNA Digestion and Ligation :

Restriction buffers supplied with enzymes were used in general except when double digests were required. Universal buffer was used for double digests.

Universal Buffer (10 x TA)

1 M	Tris Acetate pH 7.8	3.3 ml
5 M	Potassium acetate	1.3 ml
1 M	Magnesium acetate	1.0 ml
1 M	Spermidine	0.4 ml
0.1 M	DTT	0.5 ml
	H_2O	3.5 ml
		10.0 ml

(Stored at 4°C).

Ligation buffer

Tris-HCl	pH7.5	0.5 M
$MgCl_2$		0.1 M
DTT		0.1 M
Spermidine		1 mM
ATP		10 mM
BSA		1 mg/ml

(Stored at -20°C).

Calf Intestinal Phosphatase buffer (10 x concentrate)

Tris-HCl	pH9.0	0.5 M
$MgCl_2$		10 mM
$ZnCl_3$		1 mM
Spermidine		10 mM

STE buffer(10 X)

Tris-HCl	pH 8.0	0.1 M
NaCl		1 M
Na_2-EDTA		10 mM

For Eckhart Lysis:

Lysosyme (Sigma)	10^5 units (approx.)
RNAase	10^2 units (approx.)
Sucrose	20g
Tris borate buffer	100ml

For mini-preps of plasmid DNA :

Solution I

Glucose	50 mM
EDTA	25 mM
Tris-HCl pH 8.0	10 mM

Solution II - Alkaline-SDS solution

NaOH	0.2 M
SDS	1%

(Stored at room temperature for two weeks).

Solution III - High Salt Solution

Sodium acetate	3 M

STET Buffer

Sucrose	8%
Triton X100	5%
EDTA	50 mM
Tris-HCl pH 8.0	50 mM

Kirby mix

Phenol	50 ml
Chloroform	48 ml
Iso-amylalcohol	2 ml

M. 4. ANTIBIOTICS

Ampicillin, streptomycin sulphate, chloramphenicol, tetracycline, rifampacin and spectinomycin were all obtained from Sigma.

Rifampacin was freshly dissolved in a small volume of methanol and added directly to the sterilized medium at 50°C. Tetracycline was dissolved in 50% ethanol and added directly to sterilized medium. Aquous solutions of ampicillin, chloramphenicol and spectinomycin were prepared, filter sterilized and added directly to sterilized medium at 50°C. Streptomycin sulphate was dissolved in H_2O, filter sterilized and stored at 4°C until use. When necessary, the level of antibiotic to be used was determined by calculating the minimum inhibitory concentration for the particular strain.

M. 5. ENZYMES

Restriction and ligation enzymes were obtained from BRL. Alkaline phosphatase was from calf intestine by Boehringer Manheim.

M. 6. Triphosphates

dGTP, dCTP, dTTP were sodium salts by Boehringer and were diluted in H_2O to a final concentration of 10mM. ^{32}P was obtained from Amersham. A stock of 250 uCi with a specific activity of 800 Ci/mMole was obtained as required.

M. 7. CONDITIONS FOR STORING AND CULTURING BACTERIA

R.leguminosarum. R.melilotiand B.japonicum strains were stored on YMA slopes at 4°C. Deep frozen stocks of all strains were also kept by adding and equal volume of glycerol to a late log phase culture and storing at -20°C. E.coli stocks were stored on LA slopes at 4°C. In the case of hosts harbouring unstable plasmids an appropriate antibiotic was incorporated in the stock medium. Working stocks were kept on plates at 4°C. Broth cultures were grown from single colony isolates in 5 ml medium. Rhizobium require 2 days incubation at 30°C, Bradyrhizobium from 4-7 days incubation at 30°C.

E.coli was grown at 37°C and overnight cultures were diluted 1 in 10 in fresh broth and outgrowth for 2 h allowed before use.

All cultures were autoclaved before disposal.

M. 8.

Isolation of B.japonicum serogroup 123 from Soybean Root Nodules

Nodules were taken from plant roots and rinsed in water to remove soil. They were then placed in a screw cap bottle. 20 ml Tween 80 solution was added and the bottle placed on a shaker for 60 min. The Tween 80 solution was drained and replaced by a 20% chlorox solution and the bottle shaken for 45 min. The chlorox solution was drained and replaced with peroxide and the nodules shaken for a further 30 min. They were then drained and rinsed 10 times with sterile water. The nodules were aseptically removed to a microtitre tray

and crushed with a tooth-pick. The exudate was streaked on YM plates incorporating 100 ug/ml cyclohexamide and incubated for 7 days at 30°C. Single colonies of isolated strains were used to inoculate broth cultures and 1 ml of a log phase culture was mixed with half-strength molten YMA at 50°C. This soft agar was poured over a YMA base plate and when solidified the plate was spotted with 50ul of phage specific for 123, 138, and 110 serotypes. The plates were incubated at 30°C for 7 days and positive serogroup 123 isolates stored.

M. 9. ISOLATION OF ANTIBIOTIC RESISTANT MUTANTS

A late log phase culture (10 ml) was pelleted by centrifugation at 5000g for 10 min. The cells were resuspended in TES Buffer then pelleted again. The pellet was resuspended in 1 ml TES buffer and 5 aliquots of 0.1 ml plated on agar plates incorporating the appropriate antibiotic. Frequencies of mutations were estimated by serial dilution of the remaining 0.5 ml and plating 0.1 ml of the higher dilutions on medium agar plates without the antibiotic. The plates were incubated for 3 days for *Rhizobium* and 9 Days for *Bradyrhizobium*. Only 2 days incubation was required for the selection of antibiotic resistant mutants in *E.coli*. The mutants were purified out on the selective antibiotic plates .

M. 10. Bacterial Conjugation

All conjugations were performed on solid agar surfaces. Rhizobium recipients were grown in TYB, Bradyrhizobium recipients in AGB, both to late log phase. The E.coli donor strain was grown in LB to mid log phase. Donor and recipient (0.7 ml of each) were mixed in a microfuge tube, centrifuged and resuspended in 100 ul of fresh broth (TYB or YMB). The suspension was placed on a 0.45u membrane filter (Gelman) on a TYA or YMA plate and incubated overnight for Rhizobium or for 40 h in the case of B.japonicum. The filters were then aseptically removed to 5 ml of sterile water and the cells resuspended by rubbing the filter on the side of the tube and vortexing for 1 min. Dilutions were made and 0.1 ml of the appropriate dilutions were plated on antibiotic selective medium. Donor and recipient were carried through the same procedure separately and plated on selective medium to check counterselection. Transfer frequency was calculated by estimating the donor count per ml and expressing the number of transconjugants as a fraction of this.

M. 11. TRANSFORMATION

The strains routinely transformed were E.coliC600 or E.coliS17-1.

Preparation of competent cells: An overnight culture was diluted 1 in 10 in 100 ml LB and grown to O.D. A_{600} 0.4-0.6. The cells were chilled on ice and centrifuged at 4000g for

10 min at 4°C. The pellet was resuspended in 5ml of ice cold 50mM $MgCl_2$ and transferred to a pre-chilled 10 ml centrifuge tube. The cells were kept on ice for 10 min the centrifuged for 5 min at 4000g in a bench centrifuge. The pellet was resuspended in 3 ml ice cold 50 mM $CaCl_2$ and kept on ice for 45 min. The suspension was again centrifuged for 5 min and the cells resuspended in 2 ml of ice cold 50 mM $CaCl_2$. At this stage the cells were considered competent.

Uptake of plasmid DNA: DNA, usually about 1 ug dissolved in 20 ul of TE buffer, was added to 0.2 ml cold competent cells. The transformation mix was held on ice for 30 min then transferred to a 42°C waterbath for a 2 min heat shock. LB (0.7 ml) was then added and growth for 30 min at 37°C permitted. The cells were centrifuged at 4000g for 3 min and resuspended in the 50 ul of LB remaining when the supernatant had been poured off. This 50 ul was plated on selective medium. Transformants appeared after overnight incubation at 37°C. DNA (20 ul) added to 0.2 ml 50 mM $CaCl_2$ and 20ul TE buffer added to 0.2 ml competent cells were carried through the procedure and plated to check sterility and counter selection.

M. 12. RAPID PLASMID DETECTION

The following method was derived from the original method of Eckhardt (1978) with adaptations personally communicated to me by M. O'Connell (N.I.H.E., Dublin).

Sample preparation: a culture was grown to O.D.A600nm 0.3. A 200 ul aliquot was transferred to a microfuge tube and centrifuged for 2 min at 4000g. The supernatant was removed and the pellet resuspended in 200 ul of 0.02% Sarkysoyl dissolved in TE (TE only in the case of E.coli). The cells were again centrifuged and the supernatant carefully removed. The pellet was resuspended in 20 ul of lysing solution and then quickly loaded in the gel slot and electrophoresis started. (An overnight plate culture could also be used for E.coli using a loopful of culture).

Gel preparation: a horizontal slab gel of 0.7% agarose (Sigma, Type II E.E.O.) dissolved in Tris borate buffer was poured. When the gel had solidified the backpiece behind the comb was removed by cutting the gel. The back piece of the gel was repoured with the comb in place with a solution of 0.4% agarose containing 1% SDS dissolved in Tris borate buffer. A few drops of Bromophenol blue was also added to the solution. When the gel had solidified the comb was removed and the gel flooded with Tris borate buffer which was the electrophoresis buffer. The gel was then ready for loading. Gels were run slowly (about 5 volts) for 30 min or until lysis in the slots was visible. The current was then increased to 100 volts and the gel was run for 2.5 h. It was then removed to a bath of distilled water containing 5 ug/ml ethidium bromide (Sigma) and stained for 20 min. The gel was rinsed in tap water and DNA bands were visualised over a U.V. transilluminator (U.V.P. Inc., San Gabrielle, California, U.S.A.). Photography was with a Polaroid M.P. 4

Land Camera with red filter (25 A KODAK) using Polaroid 667 positive film and an exposure of 0.36 mm for 0.25 sec.

M. 13. RAPID PLASMID PREPARATION

Two techniques were personally communicated to me by Dr. Micael O'Connell and Dr. Thecla Ryan for the isolation of plasmid DNA for subsequent restriction and transformation. The first method gave DNA which was not denatured. The second method is somewhat quicker and gives higher yields but it denatures the DNA.

Method 1: AKLKALINE-SDS LYSIS

Solutions I, II and III are described in M. 3.

An overnight culture (1.5 ml) which had been grown under antibiotic selective pressure, when neccessary, was transferred to a microfuge tube and centrifuged for 2 min. The supernatant was removed and a further 1.5ml of culture pelleted. The supernatant was carefully removed and the pellet resuspended in 100 ul of solution I. The tube was placed on ice for 5 min and then solution II (200 ul) was added with gentle mixing. The tube was then placed on ice . Cell lysis could be checked at this time by the appearance of slight viscosity in the tube. After 10 min incubation on ice 150 ul of solution III was added and the tube was placed on ice again for a further 10 min. At this stage chromosomal DNA and cell debris was visible. The tube was centrifuged for 10 min in a microfuge and a 400ul aliquot of the clear supernatant was transferred to a new microfuge tube

containing 400ul of Kirby mix. This solution was vortexed for 10 sec, then centrifuged for 2 min. The upper phase was removed to a new microfuge tube and after the addition of 0.8ml of cold 95% ethanol the tube was held at -20°C for 15 min. A 15 min centrifuge in a microfuge pelleted the DNA which was then washed twice with 70% ethanol and dried under vacuum. The pellet was dissolved in 50 ul of TE containing RNAase (1 unit/ul). An overnight culture scraped from an agar plate was also successfully used instead of a broth culture.

Method 2: RAPID BOILING PREPARATION (Holmes and Quigley, 1981).

A patch of growth from an overnight culture of E.coli was scooped from a selective antibiotic plate using a toothpick and then suspended in 300 ul of STET buffer in a microfuge tube. Freshly prepared lysozyme (20 ul of 10 mg/ml) was added and the tube left at room temperature for 10 min. The tube was placed in boiling water for 60 sec and then immediately removed to a microfuge for a 10 min centrifugation. The supernatant was removed and the plasmid DNA precipitated by the addition of an equal volume of isopropanol. The tube was placed at -20°C for 20 min and then centrifuged for 5 min. The isopropanol was removed by an ether wash and the pellet dried by vacuum. The DNA was dissolved in TE containing RNAase (1 unit/ml).

M. 14. ISOLATION OF BULK SMALL PLASMID DNA

The cleared lysate procedure followed by centrifugation in a CsCl gradient was used to isolate large amounts of plasmid

DNA of up to 20 Kb in size. Usually the isolation was from E.coli strains but the technique was found to be equally useful for isolating pSUP104-S12 derivatives from Rhizobium spp.

A 10 ml culture of cells grown under antibiotic selective pressure was used to inoculate a 200 ml culture in a 500 ml flask. The scaled up culture was incubated with good aeration at 37°C (30°C for rhizobia) until it reached stationary phase. Cells were pelleted by centrifugation at 5000g and resuspended in 50 ml 0.2 M Tris pH 8. The cells were again pelleted and resuspended in a small volume of 25% sucrose in 0.5 M Tris pH 8, usually less than 3 ml. The resuspended cells were transferred to 50 ml polypropelene centrifuge tubes and placed on ice for 5 min. The solution was replaced on ice after each addition. Freshly prepared lysozyme (0.6ml of 20 mg/ml solution dissolved in 0.2 M TRIS pH 8) was added and mixed gently. After 5 min, 1.2 ml of ice cold 0.25 M EDTA pH 8 was added and mixed gently. After a further 5 min 4.8 ml of lytic mix (2% v/v solution of Triton X-100 dissolved in 0.05 M Tris/0.05 M EDTA, pH 8) was added. The solution was gently mixed at intervals for 20 min, after which cell lysis could be detected by increase in viscosity. The membrane/ chromosome components of the cell were then separated from the cleared lysate by a clearing spin at 26,000 g for 40 min. Cleared lysate was decanted and used to form a CsCl gradient. To form the CsCl gradient, CsCl (9.6 g) was dissolved in 9.2 ml cleared lysate and

transferred to Quick-seal polyallomer ultracentrifuge tubes where 0.4 ml ethidium bromide (5 mg/ml in water) was then added. Density gradient seperation of pure covalently closed circular DNA was achieved by centrifugation at 150,000 g for 20 h in a Beckman ultracentrifuge Model L8-M.

The higher density cccDNA which was visualised by a U.V. lamp MODEL Mini UVIS by Desega, Heidelberg was removed by piercing a hole in the top of the tube then inserting a 21 Gauge syringe needle just below the desired band and withdrawing the fluorescent DNA. Ethidium bromide was removed from the solution by extracting several times with an equal volume of isopropanol which was saturated with 20 x SSC. CsCl was removed by dialysis against TE (4 x 1 litre changes at 4^{o}C). The DNA concentration was calculated by reading the O.D. of a diluted sample at 260nm in a PYE UNICAM spectrophotometer in quartz cuvettes .

M. 15. ISOLATION OF TOTAL DNA FROM BRADYRHIZOBIUM STRAINS

A late log phase culture (10ml in AGB) was pelleted by centrifugation at 4000 g. The pellet washed in 3 ml TES buffer resuspended in 3 ml of 25% sucrose in TE buffer and again centrifuged. The supernatant was carefully removed and the pellet was resuspended in 600 ul of 25% sucrose in TE buffer. A freshly prepared lyzozyme solution (120 ul of a 5 mg/ml solution in H_2O) and 480 ul of 0.1 M EDTA pH 8 were added. The suspension was left at room temperature for 10 min then 1.2 ml of a 0.2% SDS solution and 300 ul of pronase (1 mg/ml dissolved in TES buffer) were added. The

suspension was incubated at 37°C for 1 h and then extracted with 2.7 ml phenol. The tube was mixed by inversion for 10 min and was then centrifuged at 5000g for 10 min. The upper phase including the white protenaceous interphase was removed to a new centrifuge tube and the phenol extraction repeated. The supernatant was then removed to a corex tube leaving behind the white interphase and centrifuged for 15 min at 27,000g. The supernatant was removed to a new tube and extracted with 2.7ml Kirby mix. Following centrifugation at 5,000g for 10 min and the supernatant was extracted with 0.5ml ether to remove any remaining Kirby mix. The DNA was precipitated by adding 270 ul of 3M sodium acetate and 6ml of cold ethanol. The tube was placed at -20°C overnight. The DNA was pelleted by centrifugation at 6,000g for 10 min washed twice in 70% ethanol dried under vacuum and dissolved in 200 ul of TE.

M. 16. ISOLATION OF TOTAL DNA FROM RHIZOBIUM STRAINS

A culture was grown in 10 ml TYB to late log phase. The cells were pelleted by centrifugation at 5000g, resuspended in 10 ml TES buffer and pelleted again at 5000g. The washed cells were resuspended in 5 ml TE buffer. Lysozyme (0.5 ml of a freshly prepared solution containing 2 mg/ml in TE) was added. The suspension was left at 30°C for 20 min and then Sarkosyl ((0.5 ml of a 10% solution in TE buffer containing 5 mg/ml pronase) was added. The suspension was incubated at 37°C for 1 h. When lysis was evident 0.7 ml of 3 M sodium acetate was added followed by 2.5 ml Kirby mix. The two phases were mixed gently for 15 min and then

separated by centrifugation at 2000g for 2 min. The aqueous phase was removed to a corex tube using 1 ml micropipette tip whose tip was removed by cutting. The solution was centrifuged at 20000g for 15 min and the aqueous solution was carefully removed to a new tube where it was extracted again with Kirby mix. A chloroform extraction was followed by an ether extraction to remove residual traces of phenol. Isopropanol (5 ml at -20^{o}C) was added and the tube inverted a few times and then left at room temperature for 15 min. The DNA was pelleted by a 5 min centifugation at 5000g then washed twice in 70% ethanol and dried under vacuum. The total DNA was resuspended in 200 ul TE buffer.

M.17

TRANSFER OF DNA TO NITROCELLULOSE FILTERS BY SOUTHERN BLOTTING

Restricted total DNA was electrophoresed in a 1% agarose gel overnight, stained and photographed before blotting. A ruler was placed along one side of the gel to facilitate measurement of distance when comparing the photograph with the final autoradiograph. Before denaturation of the gel, it was neccessary to nick the large DNA molecules by immersion in 0.25 M HCl for 10 min. The gel was washed with tap water and the immersed in denaturing solution and placed on a shaking table at room temperature for 40 min. The denaturing solution was poured off and the gel washed in tap water again. The gel was placed in neutralizing solution on the shaking table and agitated for 1 h at room temperature or until the surface of the gel was below pH 8.5 (as indicated

by pH paper) . The gel was then transferred to an upturned gel base on which there was a sheet of 3 x Whatman paper which had been soaked in 20 x SSC. This paper sheet hung over the gel base at either end and dipped into a bath of 10 x SSC. Nitrocellulose paper cut to the exact size of the gel was pre-soaked in 2 x SSC until fully wetted and was then placed on top of the gel with care taken to remove any air bubbles. The well positions were marked on the nitrocellulose for later identification. Two pieces of 3 x Whatman paper soaked in 2 x SSC cut to the same size as the gel were placed on top of the nitrocellulose filter. Finally, paper towels about 10 cm high were put on top and compressed lightly with a piece of glass. The presence of cling-film around the gel ensured that the only contact between the underlying Whatman paper wick and the paper towels was through the gel. After overnight blotting the nitrocellulose was removed from the gel and soaked in 6 x SSC for 5 min then blotted dry between two pieces of filter paper. The filter was then baked for 2 h at 80^{o}C. The gel was restained to check for the full transfer of DNA to the nitrocellulose.

M. 18. NICK TRANSLATION

The nick translation reaction was set up by the addition of the following to a microfuge tube:

10 X Nick Translation buffer	4 ul
DNA	10 ul
dGTP (10 mM)	1 ul

dCTP (10 mM)	1 ul
dTTP (10 mM)	1 ul
(^{32}P) dATP (800Ci/mM)	1 ul
DNAase (0.1 ug/ml)	0.5 ul
Polymerase 1	1 ul

The DNAase was the last component of the reaction to be added. The reaction was allowed to proceed for 60 min at 16^{o}C. Seperation of labelled DNA was made on a Sephadex G-50 column (Maniatis et al., 1982). The Sephadex was equilibrated with TES buffer and poured into a disposable syringe which had been plugged with glass wool. The column was prepared by placing the syringe in a centrifuge tube and centrifuging at 1600g for 4 min in a bench centrifuge. The sephadex packed down during centrifugation and more Sephadex was added until the packed bed volume was 0.9 ml. TES buffer (0.1 ml) was added to the column and the column was centrifuged at exactly the same conditions as before. After repeating this step the column was removed to another centrifuge tube with a microfuge tube inside. The nick translation solution was added to the column in a total volume of 0.1 ml and centrifuged at 1600g for 4 min as before. The labelled probe was collected in the microfuge tube and stored at 4^{o}C until use. The column was carefully disposed of as radio-active waste.

M. 19. DNA/DNA HYBRIDIZATION

The filter unto which DNA had been blotted by Southern transfer was soaked in 6 X SSC for 2 min. The filter was

then pre-hybridized at 68°C in an air-tight plastic box containing pre-hybridization fluid (0.2 ml per cm.^{2} of filter). The pre- hybridization fluid consisted of:

6 X SSC

0.5% SDS

5 X Denhardts' Solution

100 ug/ml denatured salmon sperm

After 4 h the pre-hybridization fluid was removed and replaced by 25 ml of hybridization fluid. The hybridization fluid was the same as the pre-hybridization fluid except that it contained 0.01 M EDTA. The DNA probe was denatured by boiling for 5 min in a microfuge tube and was then added to the hybridization mix. The ait-tight box containing the hybridization mix was re-incubated for 16 h at 68°C. The filter was then removed and the hybridization mix stored for re-use or disposed of as radio-active waste. The filter was submerged in a tray containing a solution of 2 X SSC and 0.5% SDS at room temperature, where it was washed for 5 min. It was then transferred to a fresh tray containing 2 X SSC and 0.1% SDS where it was soaked for 15 min at room temperature. The filter was removed again to a plastic box containing a solution of 0.1 X SSC and 0.5% SDS. The box was incubated for 2 h at 68°C for homologous hybridization (51°C for non-homologous hybridization). After 2 h the buffer was changed and incubation carried on for a further 30 min. The filter was dried at room temperature , sealed in a heat seal bag and exposed to X-ray film in the dark at room

temperature. The required exposure time varied from 4 days to 2 weeks. The autoradiograph was developed by a 5 min immersion in developer followed by two 1 min washes in fixer.

M. 20. CLEAVAGE OF DNA BY RESTRICTION ENDONUCLEASES

Reactions were carried out the appropriate buffer for each enzyme in a microfuge tube. The DNA to be cleaved was dissolved in TE buffer. An appropriate volume of restriction buffer was added to bring the overall buffer concentration to 1 X. The reaction volume was always large enough to ensure at least a 10 fold dilution of added enzyme. Reactions were held at the appropriate temperature for the particular enzyme (depending on manufacturers specifications) which was 37^{o}C for most enzymes. Reactions were allowed to proceed for 1 h and were then stopped by a temperature shift to 65^{o}C for 10 min. Fragments were separated on a 0.7% agarose-Tris acetate gel. Gels were stained in distilled water containing ethidium bromide (10 ug/ml). Fragment visualization and photography were as described previously for plasmid bands.

M. 20. DEPHOSPHORYLATION OF VECTOR DNA

Restricted plasmid DNA was ethanol precipitated and washed twice in 70% ethanol. The DNA pellet was dried and resuspended in 50 ul of sterile water. Dephosphorylation reactions were carried out in a total volume of a 100 ul with 10 ul CIP buffer (10 X) and 1 ul of calf intestinal phosphatase enzyme. The reaction was allowed to proceed for

30 min at 37°C, when a further 1 ul of enzyme was added. To stop the reaction 20 ul of STE buffer (10 X), 20% SDS (5 ul) and 20 ul of EDTA(100 mM) were added along with 60 ul of H_2O. The tube was held at 68°C for 15 min. The solution was extracted twice with phenol and then with ether. The DNA was precipitated by adding 20 ul of 3 M sodium acetate and 400 ul of ethanol and leaving at -20°C for 1 h.

M. 21. LIGATION OF DNA FRAGMENTS

After the restriction of target and vector DNA, the restriction enzymes was denatured by heating to 65°C for 10 min. An equal volume of Kirby mix was added and the two phases mixed well. The tubes were centrifuged to separate the two phases and the aquous layer was transferred to a new tube where the remaining Kirby mix was removed by an ether extraction. The solution was made 0.3 M with respect to sodium acetate and 2 volumes of 95% ethanol were added. The tube was placed at -20°C for 20 min then centrifuged at 26,000g for 15 min. The pellet was washed in 70% ethanol and then dried under vacuum. The DNA was resuspended in TE buffer. (DNA which had been dephosphorylated was resuspended in TE buffer also). The vector and target DNAs were mixed in the desired ratios in a volume of 17 ul. The solution was heated to 55°C for 5 min to linearize any recirculized DNA, then placed on ice. Ligation buffer (2 ul of 10 X) and 5 units of T4 ligase were added. The total volume was 20 ul. The reaction was incubated at 16°C for 16 h in a heated water bath in a cold room. Immediately before transformation

the DNA was heated to 55^{o}C to separated any cohesive ends that were not ligated. If DNA which had been dephosphorylated was being used a control of the dephosphorylation reaction was performed. The dephosporylased vector was religated to itself and transformed. If no transformants were recovered the reaction was successful.

INTRODUCTION TO RESULTS

IS elements have been isolated from R.meliloti. R.lupini. and B.japonicum strains. Some IS elements have been found clustered around the nif regions and they are thought to be involved in genomic re-arrangements, deletions and insertional mutations. Geneomic rearrangements have been observed in Rhizobaciae and therefore IS elements could be of major evolutionary significance. The introdution of the S12 gene (described in the introduction) into R.meliloti 2011, R.leguminosarum 897 and two B.japonicum strains 123 S.D. and 123 St.P. was undertaken. IS elements were subsequently isolated from R.meliloti and R.leguminosarum. Results from DNA/DNA hybridization studies using isolated IS elements as probes to analyse interstrain relationships are described.

RESULTS

R.1 TRANSFORMATION OF E.COLI S17-1 BY pSUP104-S12

The plasmid pSUP104-S12 was isolated by the rapid alkaline-SDS technique from CSH52 pSUP104-S12. E.coli S17-1 was transformed by pSUP104-S12. Transformants were selected for on LA incorporating tetracycline(10 ug/ml). A single transformant was purified and used in subsequent matings.

R.2 ISOLATION OF SPONTANEOUS MUTANTS TO RIFAMPACIN

Chromosomal resistance to rifampacin was required for the selection of rhizobia in conjugation. Table 1.1 shows the frequency at which rifampacin resistant mutants occured in different rhizobia.

R.3 INTRODUCTION OFpSUP104 INTO RHIZOBIUM AND BRADYRHIZOBIUM

The plasmid pSUP104 was mobalized by conjugation from E.coli S17-1 pSUP104-S12 to R.meliloti 2011 $rif^r str^r$ and R.leguminosarum 897 $rif^r str^r$. Matings were performed on 0.45 u membrane filters on TYA and selection for inheritance of pSUP104-S12 was made by the incorporation of tetracycline (10 ug/ml) on TYA. The rifampacin resistant rhizobia were selected by incorporating 100 ug/ ml rifampacin in the medium. Tetracycline resistant clones arose after three days incubation. It was important to incubate the plates wrapped in foil to prevent exposure to light as both antibiotics were light sensitive. The mobilization frequencies of pSUP104-S12 from E.coli S17-1 to the rhizobium strains are shown in Table 1.2. A single transconjugant from each strain was purified and used in subsequent research.

Table 1.1

FREQUENCY OF MUTATION TO RIFAMPACIN RESISTANCE

STRAIN	Frequency of mutation to rif^r
R.meliloti 2011 str^r	2.8×10^{-7}
R.leguminosarum 897 str^r	1.4×10^{-7}
B.japonicum 123 St.P.str^r	0.8×10^{-7}
B.japonicum 123 S.D. str^r	1.7×10^{-7}

Table-1.2

MOBILIZATION FREQUENCIES OF pSUP104-S12 FROM E.COLI S17-1

Recipient strain	Mobilization frequency
R.meliloti 2011 rif^r str^r	1.52×10^{-3}
R.leguminosarum 897 rif^r str^r	7.4×10^{-3}
*B.japonicum*123 St.P. rif^r str^r	----
*B.japonicum*123 S.D. rif^r str^r	----

The plasmid pSUP104-S12 was mobilized from E.coli S17-1pSUP104-S12 to B.japonicum 123 S.D. $rif^r str^r$ and 123 St.P.$rif^r str^r$. Matings were performed on membrane filters on YMA. Selection for inheretance of pSUP104-S12 was made by the incorporation of 100 ug/ml tetracycline in AGA with rifampacin (100ug/ml) incorporated for the counter selection of B.japonicum strains. No transconjugants appeared after two and three weeks incubation. This conjugation experiment was repeated several times, increasing recipient counts and varying other parameters. No transconjugants were recovered.

R.4 STREPTOMYCIN SENSITIVITY OF RHIZOBIUM CONTAINING pSUP104-S12

R.meliloti 2011rif^r and R.leguminosarum 897 rif^r transconjugants containing the plasmid pSUP104-S12, were streptomycin sensitive where the parent strains were streptomycin resistant. Tranconjugants streaked on TYA incorporating streptomycin(350 ug/ml) and tetracycline (10 ug/ml) took three days longer to grow than transconjugants streaked on streptomycin alone. The sensitivity is plasmid related, as shown by the reaquisition of resistance to streptomycin by transconjugants when positive selection for the maintenace of the plasmid by the incorporation of tetracycline was not made. Transconjugants, when selection was made for the maintenance of pSUP104-S12, were able to overcome the streptomycin sensitivity at a low level taking longer to form colonies.

R.5

ISOLATION OF STREPTOMYCIN RESISTANT RHIZOBIUM CONTAINING pSUP104-S12

Rhizobium which had overcome the dominance of the streptomycin sensitivity gene 'S12' carried on the plasmid pSUP104-S12, were selected for by plating 0.1 ml of culture at an approximate density of 10^8 cells/ml on TYA containing streptomycin (350 ug/ml) and tetracycline (10 ug/ml) .Cells were washed with sterile distilled water before plating to remove any remaining media. Tetracycline was incorporated into all media for the growth of Rhizobium carrying pSUP104-S12 to ensure stable maintenance of the plasmid. Streptomycin resistant clones arose after 3 days incubation. The parent strains without the plasmid were plated of TYA and TYA containing streptomycin (350 ug/ml). The strains containing the pSUP104-S12 plasmid were plated on TYA containing tetracycline (10 ug/ml) and TYA incorporating streptomycin to check that streptomycin sensitivity was plasmid related. Frequencies of reversion to streptomycin resistance are shown in Table 1.3.

The fact that sensitivity to streptomycin still exists in the Rhizobium strains without the selection for the pSUP104-S12 plasmid suggests that the pSUP104-S12 plasmid is relatively stable without positive selection and therefore the dominance of the S12 gene still exists.

Table 1.3

FREQUENCIES OF REVERSION TO STREPTOMYCIN RESISTANCE

Strain	Frequency of reversion to str^r TYA[str 350 ug/ml]	TYA str end tet.
R.leguminosarum 897 pSUP104-S12	1.08×10^{-3}	3.18×10^{-4}
R.meliloti 2011 pSUP104-S12	3.2×10^{-2}	1.2×10^{-2}

TABLE 1.4

FREQUENCY OF ENLARGEMENTS OF pSUP104-S12

Plasmid host strain	Frequency
R.meliloti 2011 $rif^r str^r$	4×10^{-1}
R.leguminosarum 897 $rif^r str^r$	2×10^{-2}

R.6 DETECTION OF INSERTIONS INTO pSUP104-S12

The streptomycin sensitivity in the pSUP102-S12 carrying Rhizobium caused by the dominance of the S12 gene product was inactivated insertion of IS elements into the S12 gene. Such events are selected by isolating streptomycin resistant clones. In well lysis by the modified method of Eckhardt followed by the selective migration of covalently closed circular plasmid molecules through an agarose gel was used to examine the pSUP104-S12 plasmid from the streptomycin resistant clones. The migration of DNA is inversely proportional to the mass of the molecule and by these means some of the pSUP104-S12 derivitives were seen to be larger than the pSUP104-S12 parent plasmid. Frequencies of enlargement of pSUP104-S12 that give rise to streptomycin resistance are shown in Table 1.4. Some of the streptomycin resistant clones showed little or no difference in their pSUP104-S12 plasmid. Streptomycin resistance was by some other means probably a point mutation in the 'S12' gene. Frequencies of transposition of the IS like elements into the 'S12' gene was calculated as:

Frequency of transposition = Frequency of enlargement of pSUP104-S12 X Frequency of reversion to streptomycin resistance while maintaining the pSUP104-S12 plasmid (tc^r).

The frequency for transposition of the IS like element from R.meliloti 2011 was approximately 4.8×10^{-3}. The frequency of transposition for the IS like element from R.leguminosarum 897 was approximately 1.5×10^{-5}.

R.7 ISOLATION OF pSUP104-S12 DERIVATIVES FROM RHIZOBIUM

Cleared lysates were prepared form R.meliloti and R.leguminosarum clones which showed enlargements in pSUP104-S12. Pure plasmid DNA was prepared by Cesium-Chloride-density gradient ultracentrifugation. The pSUP104-S12 derivatives from R.meliloti were restrictable but those from R.leguminosarum were not. For ease of isolation and maintenance the plasmids were transformed into E.coli S17-1 from where they were repeatedly isolated and purified by the cleared lysate density gradient method.

R.8 RESTRICTION ANALYSIS OF pSUP104-S12 DERIVATIVES

Four derivatives of pSUP104-S12 isolated directly from R.meliloti 2011 were examined. Each contained a 1.25 Kb insert in the S12 region. As a result of restriction analysis these inserts were thouht to be identical. No recognition sites for the restriction enzymes EcoR1, Pst1, BamH1, HindIII, Sal1, PvuII, Xba1, Sma1, Apa1 and Kpn 1 were found. There were sites for the restriction enzymes Dde1, Rsa1, and Sau 3A all of which have a four base pair recognition site, though the number and position of sites could not be determined because of the small size of the fragments. Upon transformation into E.coli S17-1 some of the pSUP104-S12 derivatives were seen to have deletions in the pSUP104 -S12 plasmid (pSUP104-S12::ISRm2a) (plate 1 and 2). Two derivatives of pSUP104-S12 from R.leguminosarum 897 were examined by restriction analysis on isolation from E.coli S17-1. One had a 2.7 Kb insert in the S12 region of the

plasmid. The insert had no recognition sites for the restriction enzymes EcoR1, HindIII, BamH1, Pvu II, Xba1, ApaI, and Kpn1. It contained two restriction sites for Pst1 and one for Sal 1. A proposed restriction map is shown in Figure 3. There were sites for the restriction enzymes Dde1, Rsa1, and Sau3A but the number and position of the sites could not be determined as a result of the small size of the fragments. Rsa1 lacked any sites in a 1.5 Kb region of the IS like insert. The other pSUP104-S12 derivative examined shows a deletion in the S12 gene (pSUP104-S12::ISR11a (Plate 1 and 2).

From the restriction analysis of the pSUP104-S12 derivatives the position of insertion in the plasmid was located.Figure 4

The nomenclature of IS elements is based on the strain from which they were isolated, the site of insertion if known must be indicated and a numeral given to the element to differentiate between it and any other IS element isolated from the same strain (Cambell et al., 1977).

The 1.25 Kb insert in the S12 gene isolated from R.meliloti 2011 is referred to as pSUP104-S12::ISRm2 or ISRm2 to distinguish it from ISRm1 a 1.4 Kb IS element isolated by Ruvkun (1980).

The 2.7 Kb insert in the S12 gene carried on the plasmid pSUP104 isolated from R.leguminosarum 897 is referred to from now as pSUP104-S12::ISR11 or ISR11.

Plate 1. Restriction digests of pSUP104-S12, pSUP104-S12::ISRm2 and pSUP104-S12::ISR11. (For legend see over).

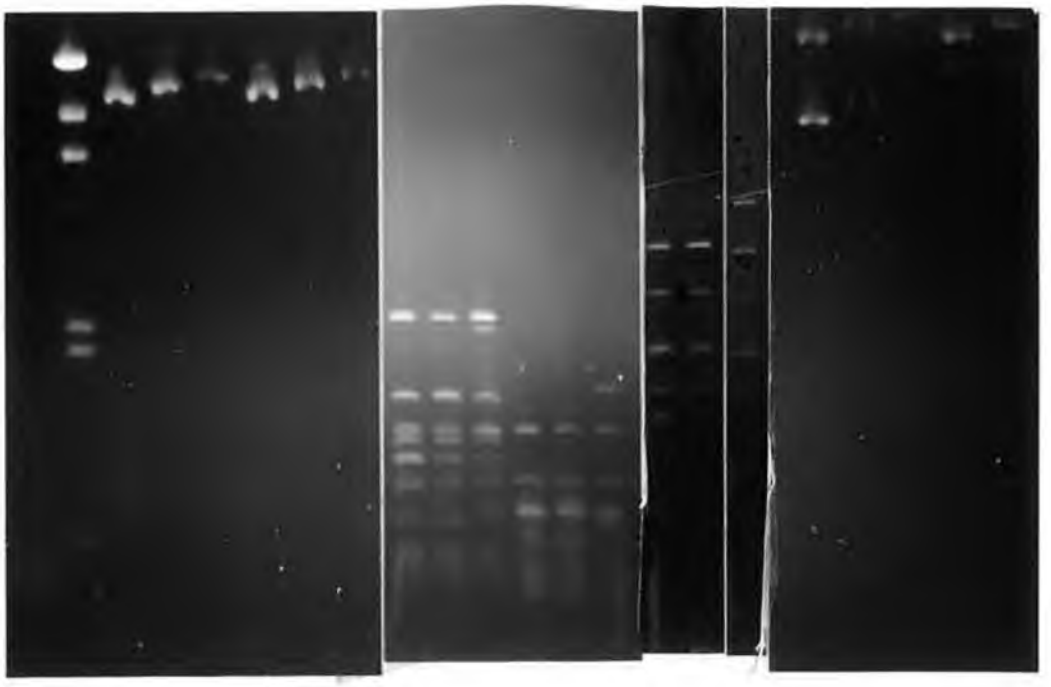


Plate 2. Restriction digests of pSUP104-S12, pSUP104-S12::ISRm2 and pSUP104-S12::ISR11 (For legend see over).

Samples shown in Plate 1 are in lanes as follows:

Lane 1:	λ	HindIII
" 2:	pSUP104-S12	EcoR1
" 3:	pSUP104-S12::ISRm2	"
" 4:	pSUP104-S12::ISRl1	"
" 5:	pSUP104-S12::ISRm2(a)	"
" 6:	pSUP104-S12::ISRl1(a)	"
" 7:	pSUP104-S12	Sal1
" 8:	pSUP104-S12::ISRm2	"
" 9:	pSUP104-S12::ISRl1	"
" 10:	pSUP104-S12	Pst1
" 11:	pSUP104-S12::ISRm2	"
" 12:	pSUP104-S12::ISRl1	"
" 13:	pSUP104-S12	BamH1
" 14:	pSUP104-S12::ISRm2	"
" 15:	pSUP104-S12::ISRl1	"
" 16:	pSUP104-S12	HindIII
" 17:	pSUP104-S12::ISRm2	"
" 18:	pSUP104-S12::ISRl1	"
" 19:	pSUP104-S12	Pvu1
" 20:	pSUP104-S12::ISRm2	"
" 21:	pSUP104-S12::ISRl1	"

Samples shown in Plate 2 are in lanes as follows:

Lane 1:	λ	HindIII
Lane 2:	pSUP104-S12	Xba1
" 3:	pSUP104-S12::ISRm2	"
" 4:	pSUP104-S12::ISRl1	"
" 5:	pSUP104-S12	Sma1
" 6:	pSUP104-S12::ISRm2	"
" 7:	pSUP104-S12::ISRl1	"
" 8:	pSUP104-S12	Dde1
" 9:	pSUP104-S12::ISRm2	"
" 10:	pSUP104-S12::ISRl1	"
" 11:	pSUP104-S12	Rsa1
" 12:	pSUP104-S12::ISRm2	"
" 13:	pSUP104-S12::ISRl1	"
" 14:	pSUP104-S12	Sau3A
" 15:	pSUP104-S12::ISRm2	"
" 16:	pSUP104-S12::ISRl1	"
" 17:	pSUP104-S12	Ape1
" 18:	pSUP104-S12::ISRm2	"
" 19:	pSUP104-S12::ISRl1	"
" 20:	pSUP104-S12	Kpn1
" 21:	pSUP104-S12::ISRm2	"
" 22:	pSUP104-S12::ISRl1	"

Figure 3 . Restriction map of S12::ISR11 (measurements in kilobases).

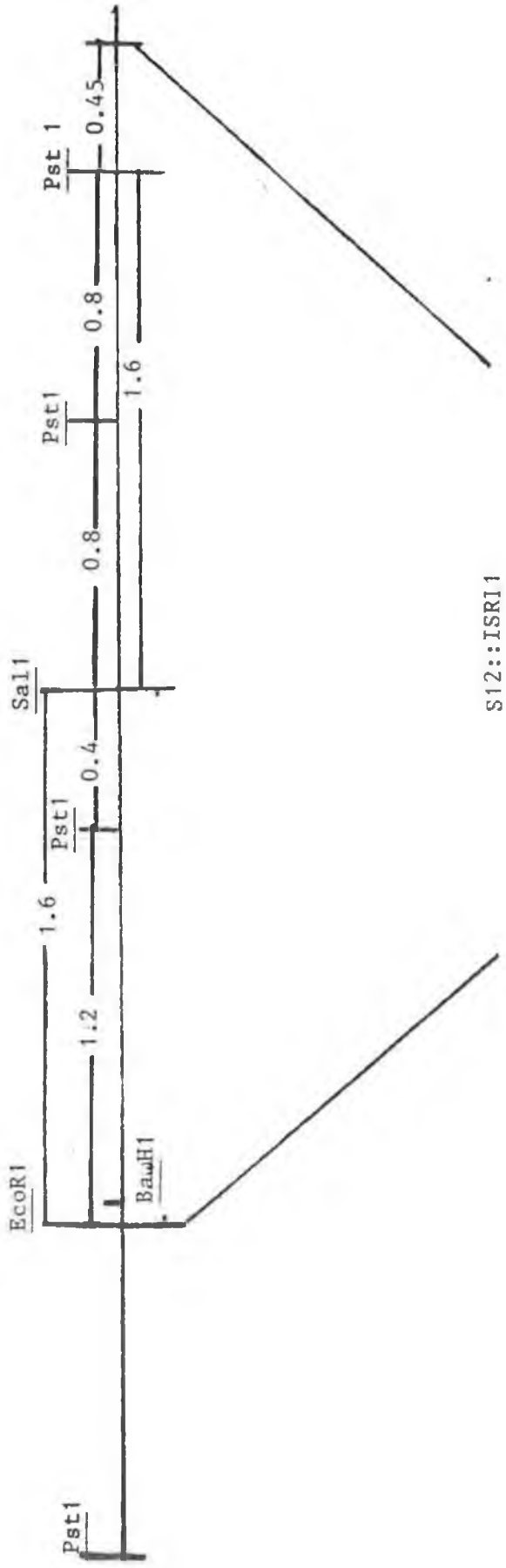
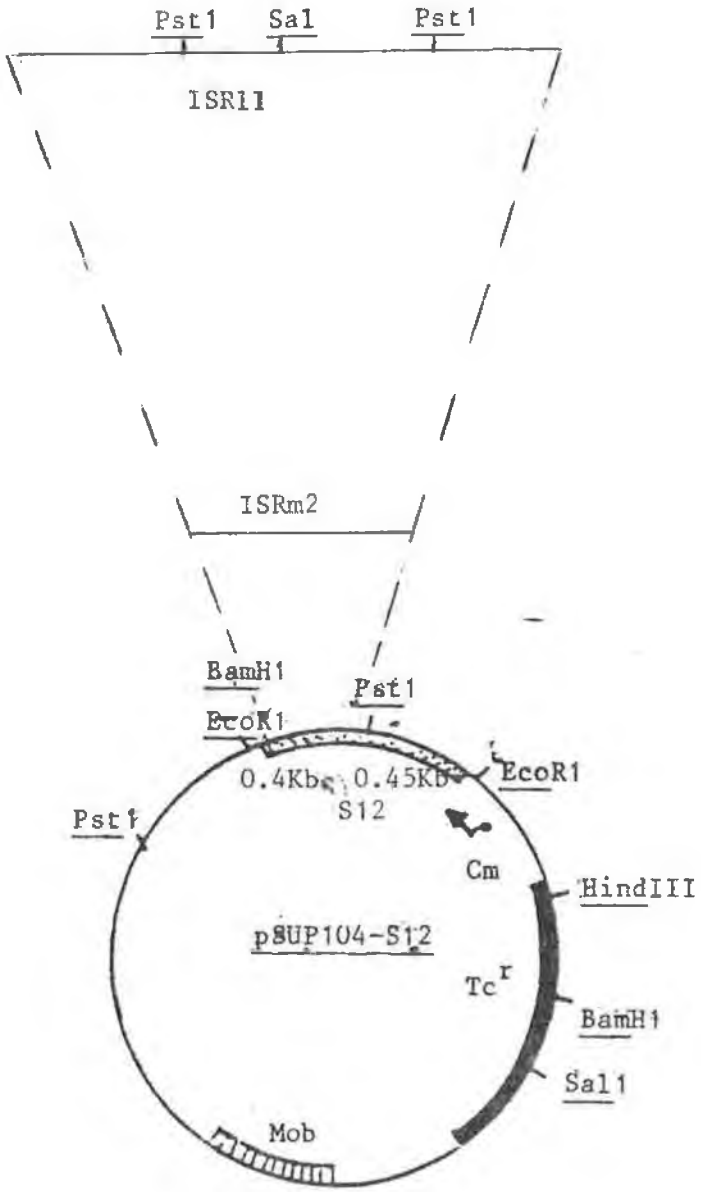


Figure 4. Location of insertion of ISRm2 and ISR11 into the pSUP104-S12 plasmid.



R.9 MOBILIZATION OF A RP4tc::pSUP104-S12 COINTEGRATE INTO B.JAPONICUM

In order to introduce pSUP104-S12 into B.japonicum an attempt was made to construct a co-integrate with RP4Δtc. E.coli 294 RP4Δtc was transformed by pSUP104-S12 selecting for tetracycline and ampicillin resistance. E.coli 294 RP4 tc-pSUP104-S12 was then crossed by conjugation with B.japonicum 123 ST.P. rif^r str^r and 123 S.D. rif^r str^r. Spontaneous mutants of B.japonicum strains to tetracycline arose at a frequency of approximately 10^{-8}. Co-integrate formation and mobilization occured at a lower frequency than this and was not detected.

R.10 CONSTRUCTION OF pRK290-S12

The plasmid pRK290 is mobilizable to and stably maintained in B.japonicum. To introduce the S12' gene into B.japonicum an attempt was made to clone the S12 region into pRK290. A schematic outline of the construction is shown in figure 5

The plasmids pSUP104-S12 and pRK290 each have a single tetracycline resistance site. After restriction of pSUP104-S12 with EcoR1 to cut out the 'S12' gene contained in an 850bp fragment, the vector was destroyed by restriction with Sal 1. Transformation of the ligation mix into E.coli S17-1 yielded an overwhelming background on pSUP104 derivatives. Transformation selects for smaller plasmids.
The 'S12' gene was cut from pSUP104-S12 and ligated to a pSUP202 derivative restricted with EcoR1 which had its

tetracycline resistance site inactivated by insertion of chromosomal DNA into the single Sal1 site. The ligation mix was transformed into E.coli S17-1 and pSUP202 derivatives were selected for by incorporating ampicillin(25 ug/ml) into the LBA. Replica plating on LBA containing ampicillin (25 ug/ml) and ampicillin with chloramphenicol(50 ug/ml) was used to select for pSUP202 clones with insertions in the Ecor1 site. Different ratios of DNA concentrations were used but the most successful, yielding an insertion frequency of 7×10^{-2} was using 10:1 ratio of pSUP104-S12 to pSUP202 . Restriction analysis showed the inserts to be the 'S12' gene. Plate 3.

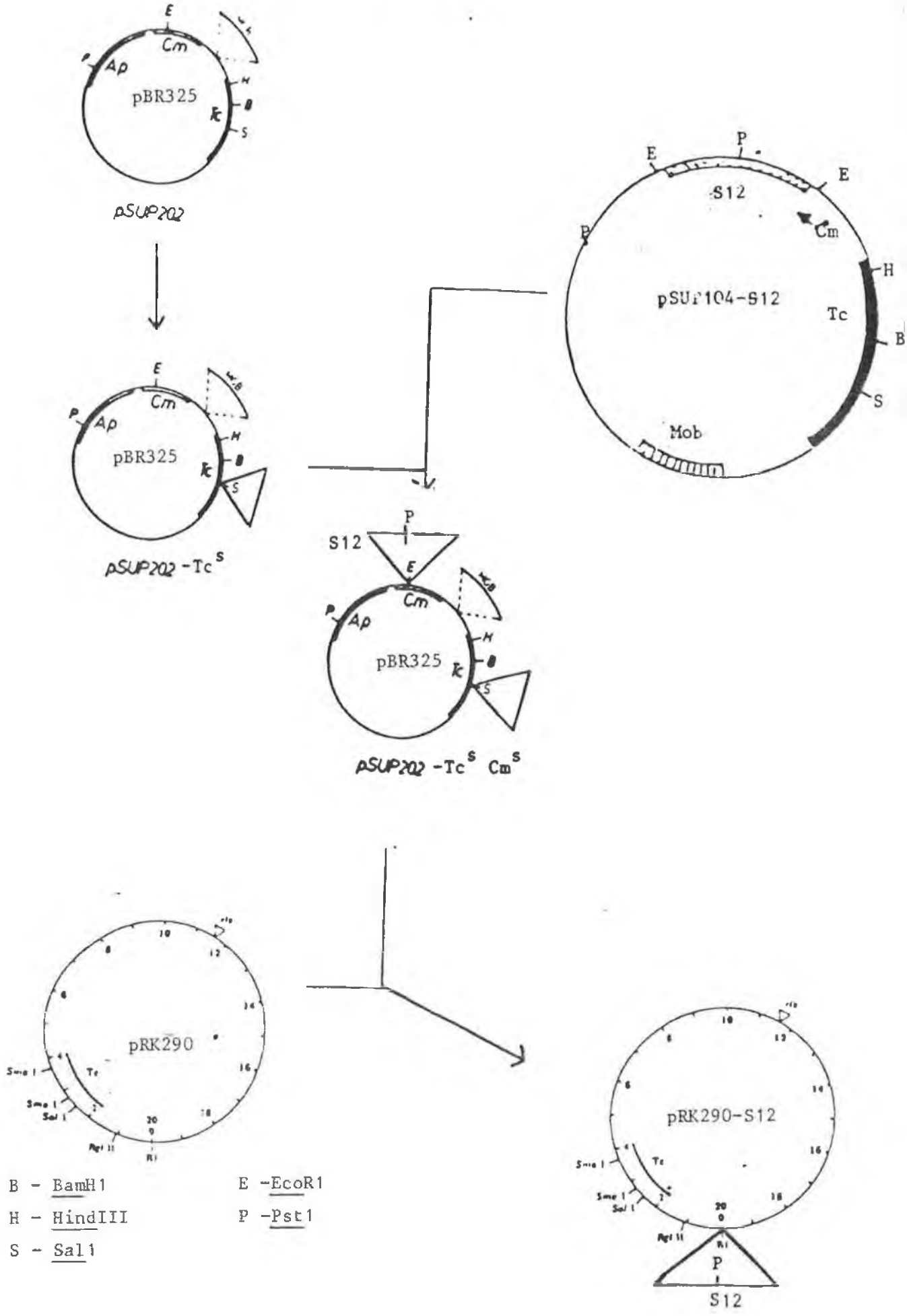
The plasmid pSUP202-S12 was restricted with EcoR1 and HindIII to cut out the S12 gene and destroy the vector. It was then ligated to pRK290 which had been cut with EcoR1 then treated with alkaline phosphatase. The ratio of pSUP202-S12 to pRK290 was again 10:1. The ligation mix was transformed into E.coli C600 str^r and selection for pRK290 clones made by incorporating tetracycline (10 ug/ml) in the LBA. A control of the alkaline phosphatase reaction was made by ligating pRK290, which had been treated with the enzyme, to itself. No transformants were detected when pRK290 (alkaline phosphatased) was transformed into E.coli C600str^r. Detection of S12 inserts in pRK290 was made by replica plating on LBA incorporating tetracycline (10 ug/ml) then LBA incorporating tetracycline with streptomycin (150 ug/ml). Sensitivity to streptomycin was shown by delayed growth in some isolates. Complete inhibition of growth was

not found. The plasmids pSUP202-S12 and pSUP104-S12were used to transform E.coli C600str^r as a control of streptomycin sensitivity. Transformants failed to grow after 2 days incubation on LBA (streptomycin(150 ug/ml) and tetracycline(10 ug/ml)). Some growth was noted after prolonged incubation but this could be due to the inactivation of the antibiotics. Rapid preparation of plasmid DNA from the pRK290 isolates showing some streptomycin sensitivity showed a pRK290 plasmid which had a recognition site for the restriction enzyme Pst1 not normally found on pRK290, though restriction with EcoR1 did not reveal an S12 fragment. On purification the streptomycin sensitivity was lost and after large scale isolation of plasmid DNA no recognition site for Pst1 was found. Plate 3 shows an example of the restriction sites of the pRK290 clones. The cloning experiment was repeated several times and the same instability of the streptomycin sensitive clones was found.

R.11 ISOLATION OF B.JAPONICUM SEROGROUP 123 FROM SOYBEAN NODULES

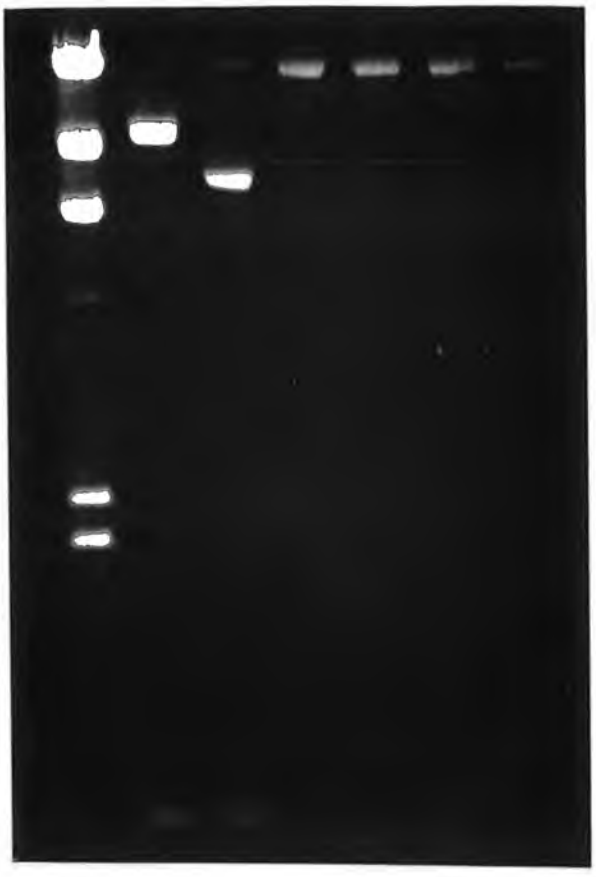
B.japonicum serogroup123 was isolated from soybean nodules from plants grown in Wisconsin soil. A single colony from each isolate was purified and used to inoculate a 10 ml broth culture. A 1 ml aliquot from each culture was used to seed a YMA plate (described in M. 8). Phage specific for serogroups 110, 123 and 138 was spotted on the plate. Lysis (indicated by a zone of clearing) by a phage specific for a serogroup indicated a positive isolate of that serogroup.

Figure 5 CLONING STRATEGY



B - BamH1
E - EcoR1
H - HindIII
P - Pst1
S - Sal1

Plate 3. Restriction digests of pSUP104-S12, pSUP202-S12, pRK290 and pRK290-S12.



Samples shown in plate 3 are in lanes as follows:

Lane 1:		HindIII
" 2:	pSUP104-S12	EcoR1
" 3:	pSUP202-S12	"
" 4:	pRK290-S12	"
" 5:	pRK290-S12	Pst1
" 6:	pRK290	EcoR1
" 7:	pRK290-S12	Undigested

B.japonicum serogroup 123 was isolated from 45% of the nodules screened.

R.12 LOCATION OF ISRm2 IN RHIZOBIUM BY DNA/DNA HYBRIDIZATION ANALYSIS

To locate the position and copy number of ISRm2 in R.meliloti and to investigate whether or not this IS element was shared with other Rhizobium strains and one Agrobacterium strain pSUP104-S12 was radioactively labelled by nick translation as described in M.18. This probe was then hybridized with total chromosomal DNA of Agrobacterium and fourteen Rhizobium strains including the parent strain R.meliloti 2011 all of which had been digested with EcoR1. Plate 5 represents an autoradiograph which corresponds to the gel in Plate 4. pSUP104-S12::ISR11 (Lane 1) and pSUP104-S12::ISRm2 (Lane 2) represent positive hybridization controls. ISRm2 hybridizes to fragments in R.meliloti 65 (lane 4), 65c (lane 5) and 2011 (lane 6). No hybridization was observed with A.tumefaciens 1060 or any of the R.phaseoli, R.trifoli and R.leguminosarum strains. The number of hybridizing bands and the approximate size of each band is given in Table 1.5. The size of the hybridizing band was calculated by aligning the filter on which the gel slots had been marked and the autoradiograph. The distance between the gel slots and the hybridizing bands was then directly measured. The size of DNA fragments is inversely proportional to the log of their mobility. The mobility in the gel of the Hind III fragments was plotted against their known molecular weight on semi-log paper. The presence of a

ruler in the photograph of the gel allowed the measurement of the mobility of fragments. The size of the hybridizing bands was then read directly from the graph.

ISRm2 hybridized with an 8.5 kb band in R.meliloti 65. With R.meliloti 65c which is a spontaneously cured derivative of R.meliloti 65 lacking a cryptic plasmid, it hybridizes to the same 8.5 Kb band and more intensly to two new bands at 9.6 Kb and 7.8 Kb. R.meliloti 2011 has eight hybridizing bands each of equal intensity though none corresponding to the bands of R.meliloti 65 and 65C.

R.13 THE DETECTION OF ISR11 IN RHIZOBIUM STRAINS

The plasmid pSUP102-S12::ISR11 was radioactively labelled with ^{32}P by nick translation as described in M.18. It was then hybridized with A.tumefaciens 1060 and 14 Rhizobium strains including R.leguminosarum 897 from which it was isolated. Plate 7 represents an autoradiograph which correlates to the gel shown in Plate 6. Hybridization occured between ISR11 the Agrobacterium strain and all the Rhizobium strains screened. pSUP104-S12::ISR11 (lane 1) and pSUP104-S12::ISRm2(lane 2) are included as positive hybridization controls. The intensity of the hybridizing bands varied greatly both within a strain and between strains. The size of the hybridizing band was calculated as described in R[. Table 1.6 shows the number of hybridizing bands in each strain and the size of each band.

Plate 4. EcoR1 digests of total chromosomal DNA from Rhizobium and Agrobacterium strains. (For legend, see over).

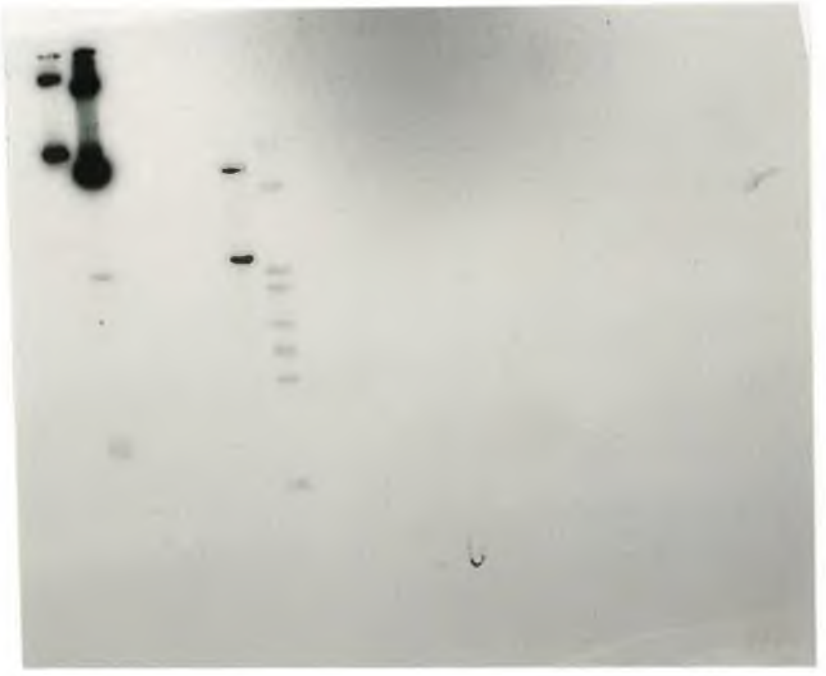


Plate 5. Autoradiograph of hybridization between ^{32}P labelled pSUP104-S12::ISRm2 and EcoR1 digests of total chromosomal DNA of Rhizobium and Agrobacterium Strains shown in Plate 4. (For legend, see over).

Samples shown in Plate 4 and 5 are in corresponding lanes as follows:

Lane 1:	pSUP104-S12::ISRl1	Undigested
" 2:	pSUP104-S12::ISRm2	"
" 3:	λ	HindIII
" 4:	R.meliloti 65	EcoR1
" 5:	" 65c	"
" 6:	" 2011	"
" 7:	A.tumefaciens 1060	"
" 8:	R.phaseoli 8002	"
" 9:	R.leguminosarum 6015pSym G1008	"
" 10:	" 897	"
" 11:	" B164	"
" 12:	" B151	"
" 13:	" 3855	"
" 14:	R.trifoli 1027	"
" 15:	" 1067	"
" 16:	" 5011	"
" 17:	" 1019	"

Table 1.5

LOCATION OF ISRm2 IN *RHIZOBIUM* AND *AGROBACTERIUM* STRAINS

STRAIN		NUMBER OF HYBRIDIZING BANDS	INTENSITY F. M. I.	FRAGMENT SIZE(Kb)
R.meliloti	65	1	1	8.5
"	65c	3	1 2	9.7,8.5, 7.8
"	2011	8	8	11,8.8,5.8,5.5 4.5,4.0,3.5,2.1
A.tumefaciens	1060	-	- - -	-
R.phaseoli	8002	-	- - -	-
R.leguminosarum	897	-	- - -	-
" pSym	8015 G1008	-	- - -	-
"	897	-	- - -	-
"	8164	-	- - -	-
"	8151	-	- - -	-
"	3855	-	- - -	-
R.trifoli	1027	-	- - -	-
"	1067	-	- - -	-
"	1019	-	- - -	-
"	5011	-	- - -	-

F = faint M = moderate I = intense.

A 9.6 Kb hybridizing band was observed in A.tumefaciens R.leguminosarum 6015pSym G1008, 897,B164, and R.trifoli 1019. An 8.3 Kb hybridizing band was observed in R.meliloti 65, 65c and 2011, R.phaseoli 8002, and R.trifoli 1027 and 1019. R.trifoli 1019 was the only strain screened which contained both the 9.6 Kb and 8.3 Kb hybridizing bands.

R.meliloti 65 contained ISR11 in the 8.3 Kb fragment where as R.meliloti 65c a plasmid cured derivative of R.meliloti 65 had the 8.3 Kb hybridizing band and two other hybridizing bands of greated intensity at 9.4 and 6.1 Kb. R.meliloti 2011 contained three hybridizing bands , one at 8.3 Kb as in the other R.meliloti strains and two others at 6.8 and 5.8 Kb.

R.leguminosarum 6015 is a derivative of strain 897 with a deletion in the symbiotic plasmid. R.leguminosarum 6015 pSym G1008 shows ISR11 located in two fragments at 9.6 and 2.15 Kb similar to those in R.leguminosarum 897 and in two new fragments at 11.5 and 6.4 Kb which were not seen in any of the other strains examined. R.leguminosarum B151, B164 and 3855 are all derivatives of R.leguminosarum 128 C53. (For clarity B151 taken from a separate gel was included in the photograph. Hybridizing bands observed with the gel from which it was taken confirmed the results found for the other leguminosarum strains). R.leguminosarum B151 is a deletion mutant of strain 3855 lacking the symbiotic plasmid pR16JI. R.leguminosarum B164 is strain B151 containing pIJ1008 the recombinant plasmid pVW5JI/pRL6JI. Of the hybridizing bands

seen in strain 3855 only one at 5.7 KB remains in the deletion mutant strain B151. This copy of ISR11 in the 5.7 Kb fragment is seen in other R.leguminosarum strains namely B164 and 897. B164 shows a new hybridizing band at 9.6 Kb on acquisition of the recombinant symbiotic plasmid pIJ1008. This band is seen in four other strains screened. One hybridizing band containing ISR11 was seen in A.tumefaciens 1060 though it does not correspond to any of the bands seen in the R.meliloti strains.R.trifoli 1027 and 1019 have multiple copies of ISR11 though R.trifoli 1067 and 5011 have only one copy in a 5.25 Kb fragment. R.phaseoli 8002 has two hybridizing bands at 8.3 and 1.7 Kb.

Plate 6. EcoR1 digests of total chromosomal DNA from Rhizobium and Agrobacterium strains. (For legend, see over).

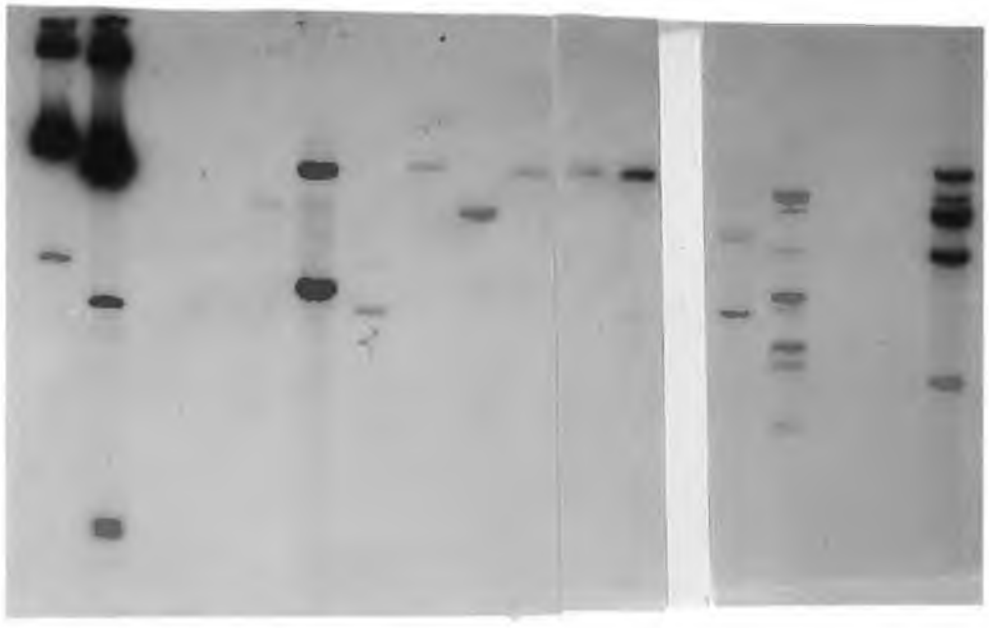


Plate 7. Autoradiograph of hybridization between ^{32}P labelled pSUP104-S12::ISR11 and EcoR1 digests of total chromosomal DNA of Rhizobium and Agrobacterium strains shown in Plate 6. (For legend, see over).

Samples shown in Plate 6 and 7 are in corresponding lanes as follows:

Lane 1:	pSUP104-S12::ISRl1	Undigested
" 2:	pSUP104-S12::ISRm2	"
" 3:		HindIII
" 4:	R.meliloti 65	EcoR1
" 5:	" 65c	"
" 6:	" 2011	"
" 7:	A.tumefaciens 1060	"
" 8:	R.phaseoli 8002	"
" 9:	R.leguminosarum 6015pSym G1008	"
" 10:	" 897	"
" 11:	" 8164	"
" 12:	" 8151	"
" 13:	" 3855	"
" 14:	R.trifoli 1027	"
" 15:	" 1067	"
" 16:	" 5011	"
" 17:	" 1019	"

Table 1.6.

LOCATION OF ISRl1 IN *RHIZOBIUM* AND *AGROBACTERIUM* STRAINS

STRAIN		NUMBER OF HYBRIDIZING BANDS	INTENSITY F. M. I.	FRAGMENT SIZE [Kb]
R.meliloti	65	1	1	8.3
"	65c	3	1 2	9.4, 8.3, 6.1
"	2011	3	2 1	8.3, 6.8, 5.8
A.tumefaciens	1060	1	1	9.6
R.phaseoli	8002	2	1 1	8.3, 1.7
R.leguminosarum	6015	4	3 1	11.5, 9.6,
pSym	G1008			6.4, 2.15
"	897	3	2 1	9.6,5.7,2.15
"	8164	3	2 1	9.6,5.7,2.15
"	8151	1	1	5.7
"	3855	6	4 2	8.7,7.8,7.5
				6.1,5.7,1.7
R.trifoli	1027	7	3 4	8.8, 8.3,6.3,
				6.05,5.1, 4.8, 3.7
"	1067	1	1	5.25
"	1019	1	1	5.25
"	5011	6	1 5	9.6, 8.8, 8.3,
				7.0, 4.55, 1.7

F = faint M = moderate I = intense.

R.14 Detection of ISRM2 and ISR11 in B.japonicum serogroup 123

B.japonicum 123 was isolated from nodules of soybeans planted in the midwestern States of the USA. Serogroup 123 is the predominant serogroup of B.japonicum found in soybean nodules. A means of identification other than serotyping is necessary to identify inoculum of serogroup 123 from indigenous bradyrhizobia. pSUP104-S12::ISRm2 which had been radio-actively labelled with ^{32}P failed to hybridize with any of the B.japonicum serogroup 123 isolates. A previous hybridization experiment showed one band of hybridization with B.japonicum 123 AR11 (shown in lane 19, Plate 9). Plate 9 represents an autoradiograph of the gel shown in Plate 8. pSUP104-S12(Lane 1) and R.meliloti 2011 (Lane 3) are included as positive hybridization controls.

pSUP104-S12::ISR11 was radio-actively labelled and hybridized with EcoR1 digests of total chromosomal DNA from fifteen B.japonicum serogroup 123 isolates. ISR11 hybridized with ten out of the fifteen isolates screened. Hybridization of pSUP102-S12 to the bradyrhizobia was not found in R.14. Plate 11 is an autoradiograph which correlates with the gel shown in Plate 10. pSUP104-S12::ISRm2 (lane 1) and R.leguminosrum 897 (lane 4) were included as positive hybridization controls. A 9.6 Kb fragment was observed in four of the B.japonicum 123 isolates which was similar to a band found in R.leguminosarum 897. B.japonicum 123 AR9 and AR6 showed similar hybridization patterns with copies of

ISR11 located in a 9.6 and 9.1 Kb fragment. B.japonicum U-123 had four bands of hybridization with ISR11. Table 1.7 shows the number and size of hybridizing bands found in B.japonicum serogroup 123 isolates with ISR11. The other B.japonicum 123 isolates showed different hybridization patterns ranging from one copy of ISR11 to four copies depending on the isolate. Some isolates contained no copies of ISR11. Hybridization of both pSUP104-S12::ISRm2 and pSUP104-S12::ISR11 to the total chromosomal DNA of the B.japonicum 123 isolates was followed by medium stringency washes at 51°C for 2 h.

Plate 8. EcoR1 digests of total chromosomal DNA of R.meliloti 2011 and B.japonicum serogroup 123 strains. (For legend, see over).

Plate 9. Autoradiograph of Hybridization between ^{32}P labelled pSUP104-S12::ISRm2 and EcoR1 digests of total chromosomal DNA of R.meliloti 2011 and B.japonicum serogroup 123 strains. shown in Plate 8. (For legend, see over).

Samples shown in Plate 8 and 9 are in corresponding lanes as follows:

Lane 1:	pSUP104-S12::ISRm2		Undigested
" 2:	λ		HindIII
" 3:	R.meliloti 2011		EcoR1
" 4:	B.japonicum 123	AR22	"
" 5:	"	AR13	"
" 6:	"	AR41	"
" 7:	"	ARL-S1	"
" 8:	"	Lamb	"
" 9:	"	Mns	"
" 10:	"	AR24	"
" 11:	"	S.D.rif^r	"
" 12:	"	AR34	"
" 13:	"	U	"
" 14:	"	AR7	"
" 15:	"	AR4	"
" 16:	"	AR14	"
" 17:	"	AR9	"
" 18:	"	AR6	"
" 19:	"	AR11	"

Plate 10. EcoR1 digests of total chromosomal DNA of R.leguminosarum 897 And B.japonicum serogroup 123 strains. (For legend, see over).

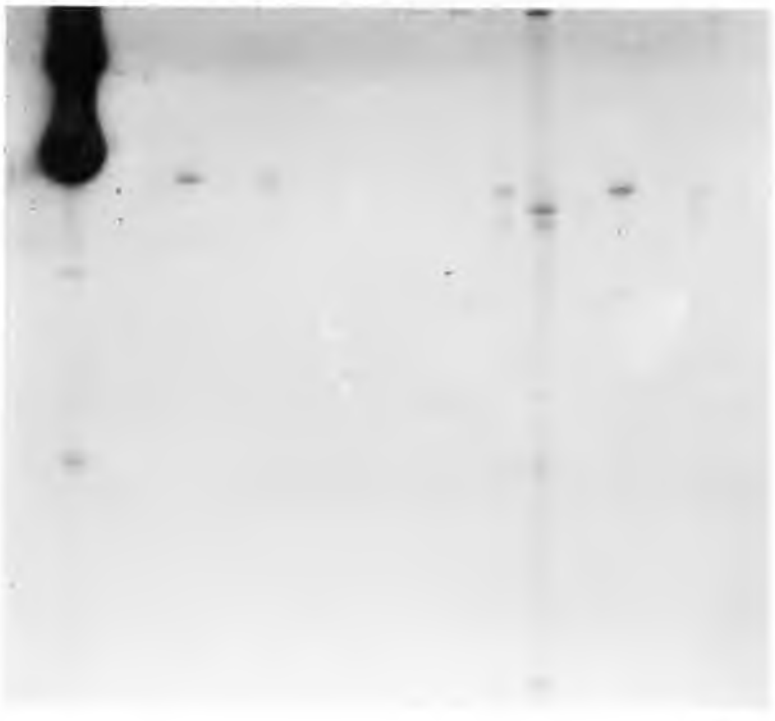


Plate 11. Autoradiograph of Hybridization between ^{32}P labelled pSUP104-S12::ISR11 and EcoR1 digests of total chromosomal DNA of R.leguminosarum 897 and B.japonicum serogroup 123 strains shown in Plate 10. (For legend, See over).

Samples shown in Plate 10 and 11 are in corresponding lanes as follows:

Lane 1:	pSUP104-S12::ISRm2		Undigested
" 2:	λ		HindIII
" 3:	B.japonicum 123	AR22	EcoR1
" 4:	B.leguminoserum	B97	"
" 5:	"	AR13	"
" 6:	"	AR41	"
" 7:	"	ARL-S1	"
" 8:	"	Lamb	"
" 9:	"	Mns	"
" 10:	"	AR24	"
" 11:	"	S.D.rif^r	"
" 12:	"	AR34	"
" 13:	"	U	"
" 14:	"	AR7	"
" 15:	"	AR4	"
" 16:	"	AR14	"
" 17:	"	AR9	"
" 18:	"	AR6	"

Table 1.7

LOCATION OF ISRl1 IN *B.JAPONICUM* SEROGROUP 123 ISOLATES.

STRAIN	NO. OF HYBRIDIZING BANDS	FRAGMENT SIZE(Kb)
B.japonicum 123 AR41	1	9.6
" " AR24	1	4.3
" " S.D. rif^r	1	8.9
" " AR34	2	9.5, 8.1
" " U	4	9, 8.4, 2.6, 0.8
" " AR7	2	9.05, 8.4
" " AR4	3	9.6, 5.7, 2.4
" " AR14	1	7.0
" " AR9	2	9.6, 9.1
" " AR6	2	9.6, 9.1

DISCUSSION

The plasmid pSUP104 was a suitable vector for the introduction of the 'S12' gene into R.meliloti 2011 and R.leguminosarum 897. The frequency of mobilization of pSUP104-S12 into R.meliloti 2011 and R.leguminosarum 897 was 1.52×10^{-3} and 7.4×10^{-3} respectively. These values were approximately a hundred fold and ten fold respectively lower than values given in the literature for mobilization of pSUP104 by E.coli S17-1 to these recipients (Simon et al., 1983; Priefer et al., 1985). The lower frequency of mobilization found in this report could be due to the selection for inheritance of the plasmid on TYA incorporating higher levels of tetracycline than given in the literature. Tetracycline (10 ug/ml) was used rather than 5 ug/ml. The presence of additional DNA encoding the 'S12' gene in the plasmid may also contribute to the lower frequencies.

The plasmid pSUP104-S12 (an Inc Q vector) was mobilized by E.coli S17-1 to B.japonicum serogroup 123 isolates, but no transconjugants were isolated despite numerous conjugations. This supports the findings of Donnelly et al.(1987) that Inc Q vectors could not be established in B.japonicum while they were readily transferred to R.meliloti strains. It is thought that the origin of replication of Inc Q vectors is unable to function in B.japonicum or is unable to function at a sufficiently high level for maintenance of the plasmid. Co-integrate formation between an RP4 plasmid which contains a deletion in the tetracycline region (rendering it

tetracycline sensitive) and pSUP104-S12 was undertaken. The subsequent transfer of the proposed co-integrate to B.japonicum 123 was attempted, as a co-integrate between RP4 and pKT230 (an IncQ plasmid) was recovered from B.japonicum after repeated attempts to introduce pKT230 by Donnelly et al.(1987). The frequency of co-integrate formation and mobilization of pSUP104-S12 and the tetracycline sensitive RP4 plasmid was lower than that of spontaneous resistance of B.japonicum 123 and no transconjugants were isolated.

The plasmid pRK290 is stably maintained in B.japonicum to which it is mobilized at a high frequency (Ditta et al., 1980). The cloning of the 'S12' gene into the single EcoR1 site of pRK290 was not directly observed. Derivatives of E.coli C600str^r isolated on LBA (streptomycin 150 ug/ml and tetracycline 10 ug/ml) Which showed inhibited growth compared to normal E.coli C600str^r pRK290 clones were seen to have acquired a pst1 recognition site, indicitive of the acquisition of the 'S12' gene. The streptomycin sensitivity ws not absolute and was shown by inhibition of growth which was overcome after one days growth. The lack of absolute streptomycin sensitivity shown when the 'S12' gene was carried on pRK290 in comparison to the total sensitivity caused by the 'S12' gene carried on pSUP104 and pSUP202 could be caused by the poor functioning of the S12 promoter or the absense of read through from the pSUP104 and pSUP202 chloramphenicol acetyl transferase promoter (M. O'Connell, personal communication).

Heteroduplex formation and rapid denaturation followed by renaturing of inverted repeats in plasmids and chromosomes has been used succesfully to detect IS elements (Chow, 1977; Ohtsubo and Ohtsubo, 1977 Schmidt et al., 1977). Other IS elements have been discovered during investigation of the mutant phenotypes of the genes in which they are inserted. More still have been found as repeated sequences in DNA/DNA hybridization studies where a section of probe has been found to hybridize to numerous bands. On investigation some repeated sequences appear to be IS elements (Kaluza et al., 1986; Rappuoli et al., 1987). The selection for inactivation of the S12 gene which confers streptomycin sensitivity on previously streptomycin resistant strains has been found to be an ideal system for the isolation of IS elements. The IS elements discovered inserted directly into the 'S12' gene into the same 400bp Pst1-EcoR1 fragment. Though streptomycin resistant with no discernable inserts in the plasmid arose, insertions were detected in between 2×10^{-1} and 4×10^{-1} of the streptomycin derivitives, which minimises the need to screen large numbers of clones. IS elements have been reported in B.japonicum (Kaluza et al., 1985), in R.lupini (Preifer et al., 1981), in R.melliloti (Ruvkun et al., 1982) and in Agrobacterium tumefaciens (Binns et al.,1982 ; Waldron and Hepburn, 1983 ; Cooksey, 1986). It is probable that other IS elements are to be found in the Rhizobaciae species. The introduction of the S12 gene into other rhizobia in a stable high copy number vector with a promoter

sequence near the cloning site would probably be fruitful in isolating other IS elements. The main drawback is finding a suitable vector for Bradyrhizobium.

ISRm2 and ISR11 have been isolated using the basic chacteristics of IS elements, that they transpose. ISRm2 transposes into the 'S12' at a frequency of 4.8×10^{-3} where as ISR11 transposes at the lower frequency of 1.5×10^{-5}. The lower transposition rate of ISR11 could be accounted for by its larger size. Transposition frequency decreases with increase in DNA size for composite elements (Chandler et al.,1981). The ends of ISRm2 and ISR11 have not been sequenced and therefore it is not known whether they contain inverted repeats characteristic of IS elements or whether they generate duplication of the target sequence on insertion. IS elements are known to cause deletions and rearrangements in plasmids even in a Rec A - background (Saedler, 1977; Iada et al.,1983). Deletions of pSUP104 and the 'S12' gene were noted in E.coli S17-1 a Rec A - strain from isolates containing ISRm2 and ISR11. The transposition activity and deletion formation, along with their location at various places in the Rhizobium genome is the basis for the classification of ISRM2 and ISR11 as IS elements.

Ruvkun et al.(1980) discovered an insertion in the fix region of R.meliloti 1021 conferring a fix - phenotype. This element was found to transpose preferentially into the Fix region at a frequency of 10^{-2}. ISRm1 was sized by heteroduplex analysis as 1.4 Kb. ISRm2 (This study) was

approximately 1.25 Kb in length (size estimate from agarose gel electrophoresis). It is possible that both elements are similar as size estimation from agarose gel electrophoresis is not totally accurate. Neither IS element have restriction sites for commonly used restriction enzymes such as HindIII, EcoR1, Pst1, BamH1, Sal1, PvuII, Kpn1, Apa 1, and Xba1. Restriction sites for Dde 1, Sau3A and Rsa1, all of which have a four base pair recognition site have been found on ISRm2. Restriction profiles for these enzymes has not been documented for ISRm1 (Ruvkunet al., 1982). ISRm1 has at least 10 copies in R.meliloti 1021 to which it was thought to be particular. Hybridization analysis of ISRm1 with other R.meliloti, R.leguminosarum and B.japonicum isolates failed to reveal any copies of ISRm1 (Ruvkun et al., 1982). ISRm1 has since been isolated from R.meliloti JJc10 where it shows no preference for insertion in the nif region (Wheatcroft and Watson, 1987). ISRm2 hybridizes to only eight bands in R.meliloti 1021 (data not shown) and to the identical eight bands in R.meliloti 2011 (R.meliloti 1021 is a derivative of strain 2011 differing only in the locus for streptomycin resistance (Meade et al.,1982)) from which it was isolated. Copies of ISRm2 were located in R.meliloti 65 and 65c and in one B.japonicum serogroup 123 isolate. Similarities exist between ISRm1 and ISRm2, both transpose at a frequency of approximately 10^{-3}. Both elements exist in high copy numbers in R.meliloti 1021. As the same strains were not used for hybridization analysis in this study as by Ruvkun et

al(1982), the main justification for the classification of ISRm2 as a separate IS element is the hybridization profile with R.meliloti 1021 (2011). No hybridization of ISRm2 with the A.tumefaciens, R.phaseoli. R.leguminosarum and R.trifoli strains and all but one B.japonicum 123 isolate was found. It is probable that as with ISRm1, ISRm2 is restricted mainly to R.meliloti strains and that it was found in B.japonicum as a result of random genetic exchange. The finding that ISRm2 along with ISRm1 is particular to R.meliloti strains adds to the bulk of serological, genetic and biochemical evidence which indicates that R.meliloti should be grouped as a separate species (Long, 1983 ; Vincent, 1981; Sadowsky et al., 1987)

ISR11 (2.7 Kb approximately) is large for an IS element which range normally from 700-2000 bp. In DNA/DNA hybridization analysis of Rhizobium strains using ISR11 as a probe, hybridization occured to bands at 2.15 and 1.7 Kb. ISR11 must therefore exist in deleted or reduced forms in Rhizobium strains. The existance of deleted IS elements has been noted in literature (Ruvkun et al., 1982 ; Meyer and Iada, 1979). Sapienza and Doolittle (1981) proposed the evolution of daughter transposons which though not self transposable can be transposed by the maternal gene products in trans. Hobo transposable elements in Dropsophilia are 3.0 Kb in size but exist in deleted non transposable forms throughout the chromosome (Streck et al., 1986). It is possible that the reduced forms of ISR11 are not

transposable. A 2.6 Kb IS element that transposes into the Ti plasmid of A.tumefaciens has been isolated (Binns et al.,1982; Waldron and Hepburn, 1983). It contains two recognition sites for the restriction enzyme Pst1 as does ISR11. It differs from ISR11 by having a recognition site for HindIII which is not found on ISR11and two sites rather than one for Sal1. The orientation of the two Pst1 sites and one of the Sal1 sites is similar to that of ISR11, and it is therefore possible that both elements are related.

IS elements are known to give elasticity to the genome, facilitating rearrangements in plasmids, insertion of plasmids into the chromosome, deletion and co-integrate formation (Reif and Saedler, 1977; Saedler et al., 1977; Shapiro et al., 1977; Kleckner, 1981; Saedler et al., 1981; Iada et al., 1983; Shapiro, 1983; Gaffney and Lessie,1987). R.meliloti 65c is a spontaneous deletion mutant of a cryptic plasmid observed in R.meliloti 65 (M. O'Connell, personal communication). Hybridization analysis of total DNA from these strains with ISR11 and ISRm2 revealed a similar pattern. The deletion mutant had gained two intense bands while retaining the original faint band. The intensity of the hybridizing band is related to the copy number of the probe in the hybridizing fragment. In the curing process it is feasible that either or both ISR11 and ISRm2 transposes into the cryptic plasmid and then by duplication inserts the cryptic plasmid into the chromosome. DNA rearrangements of this type caused by IS elements have been observed

(Kleckner, 1981 ; Iada *et al.*, 1983). Whether or not the cryptic plasmid is actually inserted into the chromosome is not known. An alternative explanation is that transposition occurs to facilitate genome adaptation during the stress put on the cell in the curing process and is not specifically involved in the loss of the plasmid. Several observations have been made of transposable elements responding to signals in their environment in such a way that transposition could preferentially occur during conditions of stress in the host (Cornelis and Saedler, 1980 ; Kleckner, 1981). Read and Johnston(1981) reported that a high proportion of *E.coli* strains that acquired one new IS element acquired a second insertion elsewhere. This could be an indication of transposition occuring in bursts in response to some signal. This could be the mechanism by which *R.meliloti* 65c acquires the new intense hybridization bands.

Both ISRm2 and ISR11 show a hybridizational similarity in *R.meliloti* 65 and 65c, though they do not hybridize to the same bands. It is not thought that the elements show sequence homology or that they are related as ISRm2 does not hybridize to any other strains where ISR11 is present. Also ISR11 hybridizes to only three bands in *R.meliloti* 2011 rather than eight for ISRm2.

Repeated sequences with IS like characteristics were isolated clustered around the *nif* region in *B.japonicum* (Kaluza *et al.*, 1985). Hybridization analysis with the IS

like elements to deletion mutants of B.japonicum revealed that some deletions directly involved the repeated sequences though some did not. R.leguminosarum B151(strain B151) and 3855 (strain 3855) are both derivatives of wild type R.leguminosarum 128C53. Strain B151 is a cured mutant lacking the symbiotic plasmid pRL6JI contained in strain 3855 (Brewin et al.,1982). Strain B151 reveals only one copy of ISR11 in the same position as one copy in strain 3855. None of the other copies of ISR11 seen in strain 3855 are maintained in strain B151. It is possible that ISR11 is involved in the curing process.

The introduction of the R.trifoli pSym G1008 plasmid into R.leguminosarum 6015 a deletion mutant of the symbiotic plasmid of strain 897 (O'Connell et al, In Press) shows one new hybridization band which is thought to be hybridization with RP4 which was used to mobalize the symbiotic plasmid G1008 into R.leguminosarum 6015. (RP4 was subsequently found to hybridize with pSUP104-S12::ISR11). The insertion of the recombinant plasmid PRL6JI/pVW5JI into the deletion mutant B151 (Gronger et al, 1987) reveals ISR11 as being located in the same position in a 5.7 Kb fragment which is shared with strain 3855, B151, 897,and 6016 psym G1008. A new copy of ISR11 in a 9.6 Kb band is found which is shared with strain 897, 6015 pSym G1008, 1060 and R.trifoli 1019. Whether ISR11 is located on the recombinant plasmid pRL6JI/pVW5JI should be investigated.

The number of copies of ISRm2 and ISR11 in Rhizobaciae

strains investigated varies from eight copies of ISRm2 in R.meliloti 2011, and at least six and seven copies of ISR11 in two R.trifoli strains to some strains only containing one copy. This leads to the possibility that transposition activity is not only controlled by the IS element or solely occuring as a response to stress but could be controlled by the host. Wheatcroft and Watson (1987) reported that ISRm1 did not transpose into the nif/fix gene region in R.meliloti JJ1c10 and suggested that the frequency of transposition into that target was host controlled.

Hybridization analysis revealed a pattern of insertion of ISR11 in some strains. In all strains except R.trifoli 1019, where ISR11 was contained in a 9.6 Kb fragment, a copy of ISR11 was not found in an 8.3Kb fragment. ISR11 was found in five strains in the 9.6 Kb fragment and in seven strains in the 8.3 Kb fragment. These bands could indicate hot spots for insertion which are mutually exclusive.

One main interest in the isolation of IS elements from Rhizobium and Bradyrhizobium strains was to investigate their usefulness in positively identifying an inoculum strain recovered from the field. B.japonicum 123 isolates some of which were isolated from the same soybean plant showed different hybridization patterns with ISR11. The hybridization pattern for some strains is the same but sufficient differences exist between others to allow the positive identification of an isolate in conjunction with

serotyping. This could be very useful in field trials of an inoculum which is of the same serotype as the indigenous population. Laboratory and field tests to prove the use of IS elements in the identification of inoculum need to be undertaken. Repeated culturing and storage of an inoculum followed by passage through the plant may lead to the alteration of the location of ISR11 in the genome. The finding that isolates of serogroup 123 differ in their is element content is not unusual. Serogroup 123 can differ in their HUP phenotypes. Serogroup 123 is also cross-reactive with other serogroups, namely 122 and 129, it is probable that ISR11 is located in these serogroups also. Hybridization patterns with ISR11 reveal that some strains are related showing ISR11 in fragments of the same size, where as some strains are not related showing no copies of ISR11 though they share the same serogroup. The isolation of other IS elements from B.japonicum using the 'S12' gene would aid the identification procedure. ISR11, the repeated sequences isolated by Kaluza et al (1986) and any newly isolated IS element would probably give a conclusive identification of any isolate.

Further research in of IS elements in Rhizobium and Bradyrhizobium could take many directions. Finding by hybridizational analysis whether ISR11 is shared among the majority of the Rhizobaciae. and clarifying its functions in genomic flexibility. The use of Eckhardt gels to examine the plasmid profiles of Rhizobium strains and their subsequent

hybridization with ISR11 would reveal whether ISR11 was carried on plasmids, especially the symbiotic plasmids. This would help to clarify its function.

Further work on deletion mutants, induced under the same conditions followed by hybridization analysis with ISRm2 or ISR11 could elucidate transposition mechanisms that lead to deletion formation and genome re-arrangements.

There is a need to construct a stable high copy number vector of low molecular weight for use in Bradyrhizobium. This vector would be needed to introduce the 'S12' gene into B.japonicum.

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