CONSTRUCTION OF A CLONING VECTOR BASED UPON A *RHIZOBIUM* 
PLASMID ORIGIN OF REPLICATION AND ITS APPLICATION 
TO GENETIC ENGINEERING OF *RHIZOBIUM* STRAINS

DISSEYATION

Presented to the Graduate Council of the 
University of North Texas in Partial 
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Pyengsoo Jeong, B.S.
Denton, Texas
May, 1992
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Rhizobia are Gram-negative, rod-shaped, soil bacteria with the ability to fix atmospheric nitrogen into ammonia as symbiont bacteroids within nodules of leguminous plant roots. Here, resident Rhizobium plasmids were studied as possible sources of components for the construction of a cloning vector for Rhizobium species. Using the pSUP202/E. coli S17-1 system, DNA segments from Rhizobium meliloti 220-13 plasmids were cloned into pSUP202. Recombinant pSUP202 molecules were conjugally transferred back into the original R. meliloti 220-13 strain. A recombinant pSUP202, carrying a segment of Rhizobium plasmid DNA encompassing five HindIII fragments (1.2, 1.3, 2.5 and two 3.2 kb fragments) was retrieved from Rhizobium conjugants via S17-1 transformation and named pPSJ123. When individual pPSJ123 HindIII fragments were subcloned into pSUP202 and conjugally transferred back to R. meliloti 220-13 under ampicillin selection, a family of related plasmids was retrieved from surviving recipient cells. Restriction analysis revealed each to carry an identical set of three "primary" HindIII fragments (1.3, 2.5
and 3.2 kb), with some carrying one or more additional fragments. These three fragments are thought to be the minimum required for stable independent replication of the molecule. Hybridization analysis showed that all of these HindIII fragments came from the second largest resident plasmid of *R. meliloti* 220-13. *R. meliloti* 220-13 could be transformed by electroporation with pPSJ123 isolated from *Rhizobium* conjugants but not with plasmid isolated from *E. coli* transformants, suggesting a restriction barrier to gene transfer may exist in this *Rhizobium* strain. The pPSJ123 conjugable *E. coli-Rhizobium* shuttle vector was utilized to introduce the parathion hydrolase gene into *R. meliloti* 220-13. However, although the recombinant plasmid was stably maintained in the strain, parathion hydrolase was not expressed at a detectable level.
ACKNOWLEDGEMENTS

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Am</td>
<td>ampicillin</td>
</tr>
<tr>
<td>BAP</td>
<td>bacterial alkaline phosphatase</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CCC</td>
<td>covalently closed circular</td>
</tr>
<tr>
<td>Cm</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>fix</td>
<td>fixation gene</td>
</tr>
<tr>
<td>Inc</td>
<td>incompatibility</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pairs</td>
</tr>
<tr>
<td>Km</td>
<td>kanamycin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>μCi</td>
<td>microcurie (10^-6 Curie)</td>
</tr>
<tr>
<td>μF</td>
<td>microfarad</td>
</tr>
<tr>
<td>Mob</td>
<td>mobilizable</td>
</tr>
<tr>
<td>nif</td>
<td>nitrogen fixation gene</td>
</tr>
<tr>
<td>nod</td>
<td>nodulation gene</td>
</tr>
<tr>
<td>ori</td>
<td>origin of replication</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>RMM</td>
<td>Rhizobium minimal medium</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Sm</td>
<td>streptomycin</td>
</tr>
<tr>
<td>Sp</td>
<td>spectinomycin</td>
</tr>
<tr>
<td>Su</td>
<td>sulphonamide</td>
</tr>
<tr>
<td>TE</td>
<td>Tris+EDTA</td>
</tr>
<tr>
<td>Tet</td>
<td>tetracycline</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylediamine</td>
</tr>
<tr>
<td>tra</td>
<td>transfer</td>
</tr>
<tr>
<td>Tp</td>
<td>trimethoprim</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>
| xg           | relative centrifugal force  
               =11.17r(rpm/1000)^2, r is a radius in cm |


INTRODUCTION

Properties of the Genus Rhizobium

Morphology of *Rhizobium*. The word *Rhizobium* is derived from the Greek words rhiza, which means root, and bios, which means life. Therefore, *Rhizobium* is a microorganism which lives in a root (Jordan 1984). *Rhizobium* is rod-shaped 0.5-0.9 μm x 1.2-3.0 μm, nonspore-forming, aerobic, Gram-negative soil bacteria (Fig. 1). However, these organisms are commonly pleomorphic (swollen, globular ellipsoidal, club shaped or branched) under adverse growth conditions. Colonies are circular, convex, semi-translucent and usually attain 2-4 mm in diameter within 3-5 days when grown on yeast mannitol mineral salts agar (Vincent 1970). Growth on carbohydrate media is usually accompanied by copious production of an extracellular polysaccharide slime. Colonies of *Rhizobium* generally absorb very little congo red dye and so remain practically colorless or only slightly pink when growing on congo red agar plates.

Physiology of *Rhizobium*. The optimal growth temperature for *Rhizobium* is 25-30°C, while the optimal pH is about 6-7. All *Rhizobium* strains exhibit host range affinities called "host specificity", based upon their nodulation activity for
Fig. 1. *Rhizobium* morphology observed under the light microscope. *Rhizobium japonicum* 191 cells cultured for one day and treated by the Gram staining procedure. Organisms are Gram-negative. 945X magnification.
the roots of specific plants (Table 1). In addition, there is a special *Rhizobium* species (*Photorhizobium thompsonianum*) which nodulates on the stem of the *Aeschynomene Indica* plant when grown under waterlogged conditions (Legocki et al. 1983).

Rhizobia can be metabolically categorized into two groups according to growth rates; fast growers and slow growers. Rhizobia isolated from legumes of temperate origin, such as *R. trifolii*, *R. leguminosarum* and *R. phaseoli*, are designated fast growers and have generation times of less than 6 hours. In contrast, slow growers (generally called *Bradyrhizobium*) such as *R. japonicum* and *R. lupini*, isolated from legumes of tropical origin, have generation times exceeding 6 hours.

All rhizobia possess the Entner-Doudoroff (ED) pathway and most hexose metabolism proceeds through the ED pathway. The tricarboxylic acid (TCA) cycle is also operational in all rhizobia. The Embden-Meyerhof-Parnas (EMP) pathway in rhizobia appears to be strain dependent. For example, phosphofructokinase activity was not detected in *R. trifolii*, indicating the lack of the EMP pathway in this species. The presence of the pentose phosphate pathway has been observed only in fast growers. Fast growing rhizobia are able to use a broad range of pentoses, hexoses, disaccharides, trisaccharides and organic acids as carbon/energy sources. On the other hand, slow growing rhizobia are unable to use disaccharides, trisaccharides or organic acids for growth.
Table 1. Rhizobium-plant interactions

<table>
<thead>
<tr>
<th>Rhizobium species</th>
<th>Host plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast-growing rhizobia</td>
<td></td>
</tr>
<tr>
<td><em>Rhizobium meliloti</em></td>
<td>Medicago (Alfalfa), Melilotus (Sweet clover),</td>
</tr>
<tr>
<td></td>
<td>Trigonella (Fenugreek)</td>
</tr>
<tr>
<td><em>R. leguminosarum</em></td>
<td>Pisum (Pea), Vicia (Vetch), Lathyrus (Sweet</td>
</tr>
<tr>
<td></td>
<td>pea), Lens (Lentil)</td>
</tr>
<tr>
<td><em>R. trifolii</em></td>
<td>Trifolium (Clover)</td>
</tr>
<tr>
<td><em>R. phaseoli</em></td>
<td>Phaeolus (Beans)</td>
</tr>
<tr>
<td><em>R. japonicum</em></td>
<td>Vigna (Cowpea), Glycine (Soybean)</td>
</tr>
<tr>
<td>Slow-growing rhizobia (Bradyrhizobium)</td>
<td></td>
</tr>
<tr>
<td><em>B. japonicum</em></td>
<td>Glycine (Soybean)</td>
</tr>
<tr>
<td><em>B. lupini</em></td>
<td>Lupinus (Lupines)</td>
</tr>
<tr>
<td>Cowpea miscellany</td>
<td>Arachis (Peanut), Vigna (Cowpea)</td>
</tr>
</tbody>
</table>

* Adapted from Alexander (1977)
However, slow growing rhizobia exhibit a relatively broad nutritional diversity with respect to aromatic growth substrates. Mannitol, the traditional carbon source for rhizobia, can give a highly variable growth response with any rhizobia, but this is particularly true of the slow growers. Gluconate has been recommended as a carbon source for slow growing rhizobia, but has given poor growth responses with cowpea rhizobia. Glycerol is the most universally used carbon source among rhizobia. However, slow growing rhizobia have a significantly longer generation time when growing on glycerol as compared to other carbon sources such as glucose, mannitol or galactose.

*R. meliloti* is restricted in its ability to utilize aromatic compounds as carbon sources. *R. meliloti* strain 102F28 is only able to utilize anthranilate, protocatechuate and the hydroaromatic compound, quinate. Other fast growing species (*R. trifolii* and *R. leguminosarum*) are able to use benzoate, catechol, *p*-coumarate, ferulate, *p*-hydroxybenzoate and protocatechuate via the β-ketoadipate pathway. The ability to use salicylate by *R. leguminosarum* and *R. trifolii* is variable and strain dependent. One strain of *R. phaseoli* was able to utilize catechol, *p*-hydroxybenzoate and salicylate. The fast growing *R. japonicum* is relatively similar to the *R. trifolii*/*R. leguminosarum* group in its ability to use aromatic carbon sources. *R. japonicum* 192 USDA is able to use benzoate, catechol, quinate,
p-hydroxybenzoate and protocatechuate, but not p-coumarate or ferulate. And the fast growing *R. japonicum* showed carbon nutritional patterns similar to other fast growing rhizobia. However, unlike other fast growing rhizobia, they were unable to utilize citrate and dulcitol as sole carbon sources (Stowers 1985).

Rhizobia do not grow well on the peptone media used routinely for culturing many bacteria. The addition of small amounts of soluble calcium salt (0.001%-0.016%) may delay or suppress the development of *Rhizobium* colonies on agar plates. Rhizobia usually grow well on TY (tryptone-yeast extract) media (Skogen-Hagenson and Atherly 1983), but cannot grow in LB and related high salt media. Elevated salt levels are toxic to rhizobia. For example, *R. trilolii* growth is significantly affected by 0.4% NaCl, while *R. japonicum* USDA 110 is inhibited by 0.6% (Yelton et al. 1983). An exception to this rule is *Rhizobium meliloti*, which grows well on LB media (1.0% of NaCl).

**Bacteroids.** Rhizobia are characteristically able to invade the root hairs of temperate zone and some tropical zone leguminous plants (family *Leguminosae*) and incite the production of root nodules (Fig. 2; Fig. 3). Here the bacteria occur as intracellular symbionts. The bacteria are present in root nodules as pleomorphic forms (bacteroids) which are normally involved in fixing atmospheric nitrogen into a combined form (ammonia) utilisable by the host plant.
Fig. 2. *Rhizobium* nodules on the roots of alfalfa. *Rhizobium meliloti* 220-13 were grown in 10 ml LB medium and mixed with surface-sterilized (ethanol-treated) alfalfa seeds. Inoculated alfalfa seeds were germinated and allowed to grow in pots containing sterile Vermiculite® under the following conditions; light period 15 hours per day, temperature 23°C and 80 watt illumination for 30 days.
Fig. 3. Nodule structure observed under scanning electron microscope. *Rhizobium japonicum* 191 were grown in 10 ml RMM media and inoculated to soybean seeds. Inoculated soybean seeds were grown for 2 months as described in Fig. 2. A. 100X magnification. B. 200X magnification
(Vincent 1981). *R. leguminosarum* bacteroids are able to use organic acids as carbon and energy sources, while *R. japonicum* bacteroids oxidize organic acids, aldehydes and alcohols. Sugars are generally not metabolized by *Rhizobium* bacteroids. Hence, organic acids are considered the primary energy source for bacteroids (Jordan 1984). *Rhizobium* bacteroids are able to store carbon as poly-ß-hydroxybutyrate and polymers of ß-hydroxybutyrate can represent up to 50% of the dry weight of *R. japonicum* bacteroids (Stowers 1985).

**Plasmids of Rhizobium**

*General characteristics of bacterial plasmids.* A plasmid is commonly defined as an autonomously replicating covalently closed circular (CCC) extrachromosomal DNA molecule (Hardy 1981). However, some plasmids found in fungi have RNA forms (Hardy 1981) and recently plasmids from a variety of *Streptomyces* species have been found to be double-stranded linear DNA molecules (Kinashi and Shimaji-Murayama 1991). Although plasmids are physically independent of the bacterial chromosome, biochemically their replication generally depends on many host-encoded proteins.

Plasmid-based phenotypes are important considerations in medicine, agriculture, commerce and the environment (Timmis and Puhler 1979). R plasmids (RP1, RP4, R68.45 etc.) can confer antibiotic resistance (e.g., ß-lactamase, chloramphenicol acetyltransferase) in pathogens of animals.
and man. Virulence plasmids (e.g., ColEl) can code for toxins (e.g., colicin) and other proteins (e.g., haemolysin, adhesion antigen) which increase the virulence of these pathogens. The Ti (tumor-inducing) plasmid in Agrobacterium tumefaciens induces a plant tumor called crown gall (cancer of the plant) in many dicotyledonous plants. This disease is agriculturally very important because it can greatly reduce yields from fruit trees and other important crops. However, the Ti plasmid has also proven to be a very useful cloning vector for plant hosts (Knauf and Nester 1982). The properties of the Ti plasmid allow new genes to be readily integrated into the plant nuclear genome (Lichtenstein and Draper 1985). Another group of agriculturally important plasmids are the Rhizobium plasmids, some of which encode genes enabling the bacteria to fix nitrogen in plant root nodules.

Some plasmids in Streptomyces species (e.g., Streptomyces coelicolor) also produce antibiotics which can be used to control pathogenic bacteria (Hopwood et al. 1979). Metabolic or degradative plasmids (e.g., the TOL, SAL and NAH plasmids) encode a wide range of metabolic activities and enable bacteria to degrade or detoxify compounds which would otherwise accumulate as deleterious organic environmental pollutants (Hardy 1981). The insecticide parathion and the herbicide 2,4-D (2,4-diphenoxacyetic acid) are also degraded by plasmid-encoded enzymes (e.g., parathion hydrolase).
Plasmids show considerable variation in overall size (ca. 3-500 kb) and copy number (ca. 1-200 copies/cell). Plasmids can be grouped into 2 categories according to whether they utilize DNA polymerase I or polymerase III as their primary DNA polymerase for replication. Many of the plasmids that use polymerase I for replication are of the small non-self-transmissible type, such as ColEI. These are often present in the bacteria as multiple copies, sometimes as many as 40-50 per cell. The replication of these plasmids is said to be under relaxed control. Another group of plasmids, usually about 30 kb or larger, utilizes DNA polymerase III for replication and commonly maintains about 1-3 copies per bacterial chromosome. The replication of these plasmids is said to be under stringent control. In the case of DNA polymerase I utilizing replication, plasmid replication may continue for some time after chromosomal replication has stopped. Plasmids that use DNA polymerase I for replication also do not appear to require the synthesis of initiator proteins prior to each new round of replication, while plasmids which use DNA polymerase III do. Taking advantage of this phenomenon, the copy number of DNA polymerase I dependent plasmids can be "amplified" to hundreds of copies/cell in the presence of chloramphenicol, an antibiotic known to be an inhibitor of protein synthesis (and which therefore blocks host chromosomal replication) in bacteria.
Bacteria often contain two or more different plasmids which can coexist in the same cell. These plasmids are said to be compatible. Plasmids which belong to the same incompatibility group cannot coexist in the same cell and after a few generations of bacterial growth, one or other is generally lost (Couturier et al. 1988). There are about 30 incompatibility groups recognized among the plasmids of enteric bacteria (Novick 1987). Among the best characterized incompatibility groups are IncF (F plasmid), IncN (pCU1), IncP-1 (RP1, RP4, R68.45, RK2), IncP-9 (pWWO), IncQ (RSF1010, R300B, pKT230) and IncW (Sa).

Plasmids can be either conjugative or nonconjugative. Conjugative plasmids can be transferred into recipient cells by self-encoded proteins and recognition sequences. They can also transfer pieces of chromosomal DNA from the donor to recipient bacterial cells. This class of plasmids (e.g., F plasmid, R 68.45, etc.) has proven to be very useful for mapping the positions of chromosomal genes.

A replicon, physically defined, consists of the DNA segment which participates in a single cycle of DNA replication. Genetically defined, a replicon consists of the DNA sequences required to specify the origin of replication and allow it to function in a regulated way, as well as any specific replication termination sequences which exist and define the process of replication (Hardy 1987).
The properties of the major classes of well characterized replicons are as follows:

1. ColEl-type replicons. The ColEl-type replicons, such as pMB1 (replicon of pBR322) and p15A (replicon of pACYC177) do not specify a plasmid-encoded essential replication protein and thus continue to replicate in chloramphenicol-treated bacterial cells (Clewell 1972). Using ColEl (6.6 kb) replication as an example, RNA polymerase transcribes a preprimer RNA (RNA II) which is processed by RNaseH to form a primer for leading strand synthesis. Regulation is via an RNA (RNA I) complementary to the preprimer. This RNA binds to the preprimer RNA, interfering with its processing. The protein product of the rom gene promotes this RNA-preprimer interaction and thus potentiates the repression of initiation (Cesareni et al. 1982).

2. RK2 replicons. RK2 (60 kb) is a broad host range Gram-negative plasmid in which direct repeat sequences adjacent to oriV (the vegetative replication origin) appear to play a part in the regulation of plasmid copy number. However, the control is apparently provided by two plasmid-encoded repressor proteins. These proteins inhibit transcription of the plasmid-encoded trfA gene, whose product is essential for replication (Stalker et al. 1981).

3. R6K replicons. R6K (38 kb) is a conjugative E. coli plasmid with a copy number of 13-40 per cell. Initiation of
R6K replication depends upon the binding of a plasmid-encoded replication protein to a series of tandem direct repeats (Kolter and Helinski 1982). The replication is controlled by the protein, acting positively at low concentrations and negatively at high concentrations. This protein also autoregulates its own synthesis (Germino and Bastia 1983).

4. F and P1 replicons. The F (100 kb) plasmid, a member of the FI incompatibility group, is best known as the prototype sex factor and has a copy number of one. P1, a nonconjugative but mobilizable large plasmid of about 90 kb, is the prophage form of a temperate bacteriophage. A number of these plasmids possess two groups of direct repeats which may bind the essential, plasmid-encoded replication protein (rep gene product). One set of repeats appears to be positively required for replication (oriV function) and for the autogenous regulation of rep. The second set appears to modulate plasmid copy number by this required positively acting protein (Lane 1981; Froehlich et al. 1983).

Plasmid replicons form the basis of the major class of cloning vectors. Therefore, determining what is necessary for stable plasmid replication is important. Much research has been carried out with the objective of isolating the smallest DNA segments capable of independent replication, called mini-replicons, for the construction of cloning vectors. For example, Lovett and Helinski (1976) isolated a single small self-replicating region from the sex factor.

However, it has become clear that although mini-replicons may retain some of the parental plasmid properties, they often lack important characteristics of the parental plasmid such as copy number control circuits. Thus, although mini-replicons may define the replication origin and essential positively acting genes, additional modulations of replication may have been deleted. For instance, large plasmids often have multiple replication genes. The RK2 (60 kb) plasmid has *trfA* (transacting replication function) and *trfB*, in addition to the origin of replication region. The
RSF1010 (8.9 kb) plasmid also has 3 replication genes (repA, repB, repC) which are located several kb distant from the origin of replication region. The F' lac plasmid is notable for its possession of 4 basic replication genes. These are RepFIA, RepFIB, RepFIC and RepFIIA (Scott 1984). The occurrence of several genes essential for replication, often physically separated from each other, makes it difficult to construct small cloning vectors from large replicons. This was in fact one of the difficulties encountered in this project.

Plasmids found in *E. coli* and related members of the Enterobacteriaceae have been the subject of most of the research on the structure and replication of plasmids. Progress in the study of these important plasmid properties accelerated markedly during recent years with the development of important technical advances for the analysis and manipulation of plasmid genes and functions. By taking advantage of these findings, new plasmids were reconstructed as cloning vectors using a variety of replicons or ori regions for the genetic manipulation of Gram-negative bacteria (Table 2) and a variety of hosts, such as plants, animals, yeast and fungi (Pouwels et al. 1987).

**Properties of Rhizobium plasmids.** Most Rhizobium strains carry large plasmids called megaplasmids, ranging in size from 100–600 kb (Burkardt et al. 1987; Rosenberg et al. 1982; Spitzbarth et al. 1979; Zurkowski and Lorkiexicz 1976). In
Table 2. Common broad host range plasmids for Gram-negative bacteria

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Replicon</th>
<th>Sizes (kb)</th>
<th>Characteristics*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural plasmids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP1, RP4</td>
<td>P-1</td>
<td>60</td>
<td>Am(^\pm), Km(^\pm), Tet(^\pm), Tra(^+)</td>
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<tr>
<td>R68.45, RK2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>RSF1010, R300B</td>
<td>Q</td>
<td>8.9</td>
<td>Su(^\pm), Sm(^\pm), Mob(^+)</td>
</tr>
<tr>
<td>Sa</td>
<td>W</td>
<td>29.6</td>
<td>Km(^\pm), Cm(^\pm), Sp(^\pm), Su(^\pm), Tra(^+)</td>
</tr>
<tr>
<td>pJP4</td>
<td>p-1</td>
<td>52</td>
<td>growth on 2,4-D</td>
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<tr>
<td>pWW0</td>
<td>P-9</td>
<td>117</td>
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<td>Cloning vectors</td>
<td></td>
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<tr>
<td>pKT230</td>
<td>RSF1010</td>
<td>11.9</td>
<td>Km(^\pm), Sm(^\pm), Mob(^+)</td>
</tr>
<tr>
<td>+ pACYC177</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>pSUP204</td>
<td>RSF1010</td>
<td>12</td>
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<td>pRK290</td>
<td>RK2</td>
<td>20</td>
<td>Tet(^\pm), Mob(^+)</td>
</tr>
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<td>pLAFR1</td>
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<td>21.6</td>
<td>Tet(^\pm), Mob(^+), cosmid</td>
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<td>pGV1106</td>
<td>Sa</td>
<td>8.4</td>
<td>Km(^\pm), Sp(^+)</td>
</tr>
</tbody>
</table>

* Inc, incompatibility; kb, kilo base pairs; Am, ampicillin; Cm, chloramphenicol; Tet, tetracycline; Sm, streptomycin; Su, sulphonamide; Sp, spectinomycin; Tra\(^+\), transfer proficient; Mob\(^+\), mobilizable; 2,4-D, 2,4-dichlorophenoxyacetic acid.
many Rhizobia, more than one large plasmid is present and consequently 10 to almost 30% of the total genetic information in such strains is plasmid-encoded. In these cases possibly some or all of the symbiotic properties are genetically controlled by these large plasmid DNA molecules. There is a good evidence that nodulating ability is encoded by plasmid borne genes (Broughton et al. 1984; Heron and Pueppke 1984). The conjugative R. leguminosarum plasmid pJB5JI (350kb) carries genes that determine the ability to nodulate peas (Beynon et al. 1980). R. japonicum USDA 191 plasmid (300kb) encodes nodulation specificity for soybean (Appelbaum et al. 1985). The nitrogen fixation (Nif) genes also appear to be plasmid borne (Dunican and Tierney 1973; Masterson et al. 1982; Prakash and Schilperoort 1982).

Melanin production encoded by a cryptic plasmid in a R. leguminosarum has also been reported (Hynes et al. 1988).

Molecular genetic studies of Rhizobium

Studies of Rhizobium genes have primarily emphasized the Nif (nitrogen fixation) genes (Fisher and Hennecke 1984; Kaluza and Hennecke 1984; Prakash et al. 1981) because of their agricultural importance. The nitrogen fixation genes of R. meliloti were mapped by using Tn5 (Ditta et al. 1980). The nod (nodulation) genes and fix (fixation) genes on the plasmid were also studied in association with the nif genes (Appelbaum et al. 1988; Christensen and Schubert 1983;
Gronger et al. 1987; Long et al. 1982; Ramakrishnan et al. 1986; Sutton et al. 1984). The nodulation promoters (nodD, nodA, nodF, nodM), in addition to nif promoter (Johnston and Downie 1984) and symbiotic promoters (Better et al. 1983) in the nodulation region of the R. leguminosarum Sym plasmid pRL1JI, were identified (Spanick et al. 1987). The hup (hydrogenase uptake) gene, whose product oxidizes hydrogen produced by nitrogenase, was also characterized (Nelson et al. 1985; Tichy and Lotz 1985; Zuber et al. 1986). The glutamine synthetase I gene of R. meliloti was studied since it encodes a major enzyme in the assimilation of ammonia (Sommerville and Kahn 1983). The recA gene of R. meliloti was isolated and characterized by the interspecific complementation of an E. coli recA mutant with plasmids containing a gene bank of R. meliloti DNA (Better and Helinsky 1983). The R. meliloti RecA protein can function in recombination and in response to DNA damage when expressed in an E. coli recA host. Hybridization studies showed that substantial DNA sequence homology existed between the recA gene of E. coli and that of R. meliloti. The DNA-dependent RNA polymerase of R. leguminosarum 300 was isolated and characterized (Lotz et al. 1981). Genes involved in lipopolysaccharide production have also been identified in the chromosome of R. leguminosarum (Priefer 1989).

Gene transfer to Rhizobium
There are 3 common types of gene transfer techniques utilized with *Rhizobium*; transformation, conjugation and transduction.

**General mechanisms of transformation.** The term transformation is used to describe the uptake of genetic information in the form of naked exogenous DNA (plasmid DNA or chromosomal DNA) into the recipient cells.

There are three basic types of transformation, classified according to the nature of the donor DNA. These are replacement, plasmid and facilitated plasmid transformation. In replacement transformation, the donor DNA is substituted for homologous sequences in the chromosome or resident plasmid of the recipient cell. Plasmid transformation occurs when a new plasmid replicon is established in a recipient cell without recombination between donor and recipient DNA. If the donor plasmid DNA contains homology to DNA (chromosomal or plasmid) in the recipient cell, recombination may participate in the establishment of the plasmid replicon. In this case, the process is called facilitated plasmid transformation (Porter 1988).

Natural transformation does not appear to be common in bacteria and most studies have used a few strains of *Streptococcus* spp., *Bacillus* spp., *Haemophilus* spp., *Neisseria gonorrhoeae* and some of the cyanobacteria (e.g., *Anacystis nidulans*). In the case of natural transformation with chromosomal
DNA, there are three general stages in the transformation process. The details of each stage differ between bacterial groups that have different cell wall structures (specifically Gram-negative versus Gram-positive bacteria).

The first stage is that of competence development, or the ability of cells to take up DNA through changes in the cell wall. This state occurs naturally in some organisms, such as Streptococcus, Bacillus, Haemophilus (natural competence), and can be artificially produced in others such as E. coli (artificial competence). Natural competency involves the formation or activation of special DNA receptor proteins. These can be induced in some Streptococcus species by polypeptide "competence factors". Cells of naturally transformable species are not always competent to take up DNA. In cultures of Streptococcus, competence develops during late logarithmic growth and Bacillus subtilis develops competence at the onset of stationary phase. In contrast to natural competency, the creation of artificial competency in E. coli (Mandel and Higa 1970) and Staphylococcus aureus (Lindberg et al. 1972) can be obtained by the treatment of cells with CaCl₂ at 0°C. In Streptococcus pneumoniae that have become naturally competent, one or more of an estimated 30 to 80 cell wall receptor sites are capable of binding double-stranded DNAs from almost any source. In contrast, Haemophilus influenzae bacteria possess a more limited number of receptors (4-8) which primarily recognize duplex DNA
carrying special 11 base pair "uptake site" sequences (5'AAGTGGCGGTCAG'). The fact that such uptake sites are especially common in Haemophilus DNA (about 600 such sequences are found in the genome) and relatively rare in other DNA's, generally restricts the Haemophilus transformation process to donor bacteria of its own genus.

The second stage, DNA binding and uptake, involves direct interaction between the plasma membrane receptors and donor DNA. This sequence of events is at first reversible, but as more cell membrane proteins become involved, DNA attachment is considerably increased. In Streptococcus and Bacillus subtilis (Gram-positive bacteria), the bound DNA double helix is nicked by endonucleases and broken into smaller pieces that average about 14 kb, which are then attacked by an exonuclease to yield internalized single-stranded molecules. In Haemophilus (Gram-negative bacteria), bound DNA is generally taken into the cell as intact double helices.

The last major stage begins with the intracellular transport of the transforming DNA in some protected form to the recipient chromosome. This DNA can be either complexed with a specific DNA binding protein or contained within small vesicles (transformasomes) derived from the recipient cell surface, or both. During the culmination of the final stage, integration, a single strand of donor DNA is incorporated into the recipient chromosome by displacing a homologous section of the recipient DNA duplex, which is then excised and
degraded. The integrated donor strand then replicates, forming a double helix (Birge 1981; Strickberger 1985). Thus, if foreign donor DNA is used which possesses little or no homology with the recipient, successful transformation will rarely take place.

In cases where transformation with nonhomologous DNA is necessary, the use of plasmids or other vectors functional in the host is the preferred method. The mechanisms of natural transformation with plasmid DNA are not well understood. Plasmid transformation may occur by the reassembly of internalized single-stranded DNA fragments, brought into the cell in a fashion similar to the mechanism for linear chromosomal fragments. For example, plasmid replicons were assembled in a recipient cell by the pairing of fragments of single strands which had entered the cell separately from duplex donors cleaved on the cell surface during Strepotococcus transformation (Saunders and Guild 1981). In N. gonorrhoeae, most of the donor plasmid DNA is cut at nonspecific sites into linear double-stranded molecules during uptake (Biswas et al. 1986), but some intact circles may also enter. Thus, either repair of linearized molecules or the occasional entry of intact circles may produce the plasmid transformants. In the case of artificial transformation (artificially-induced competence) with plasmids, transforming molecules enter the cell as intact double-stranded DNA. Although no alteration is made upon
entry, the exact method of how the plasmids cross the cell membrane as a result of artificial treatment is not known.

Transformation of *Rhizobium*. *Rhizobium* cells are not naturally competent. Therefore, several methods of producing induced competence have been developed for these bacteria. For example, Balassa (1960) transformed cysteine auxotrophic *Rhizobium lupini* with DNA from prototrophic *Rhizobium lupini* by simple incubation. Raina and Modi (1969; 1972) also transformed adenine auxotrophs of *Rhizobium* with prototrophic DNA using a competence medium. O’Gara and Duncan (1973) transformed *Rhizobium trifolii* with RP4 by simple incubation. Kiss and Kalman (1982) transformed *Rhizobium meliloti* with pKK2 (a derivatives of the wide host range plasmid Sa of the W incompatibility group) using a MgCl$_2$ and CaCl$_2$ method. Selvaraj and Iyer (1981) transformed *Rhizobium meliloti* with pRK248 using a freezing-thawing method. Bulleriahn and Benzinger (1982) also demonstrated transformation of *R. leguminosarum* with R68.45 DNA by a freezing and thawing method in the presence of CaCl$_2$ and MgCl$_2$. Protoplast (spheroplast) transformation was carried out by Berry and Atherly (1984) with *R. japonicum* using RPl and RP4. Finally, Guerinot et al. (1990) electro-transformed *Bradyrhizobium japonicum* with pRK290.

Even though the various techniques mentioned above have been utilized for the transformation of some *Rhizobium*, these techniques have not proven to be generally applicable.
to a wide variety of Rhizobium species. In this paper, several modified methods based on previous techniques were applied for the transformation of Rhizobium strains and are described below.

**General mechanisms of conjugation.** There are three types of plasmid transfer via conjugation, distinguished by the gene functions on the plasmids involved. First, F, R68.45 and RP4 plasmids encode the tra function (the ability to make the transferring apparatus, pili and an endonuclease which can cut at the oriT site) and an oriT site (or mob site). This group of plasmids is self-transmissible. The second type of transfer is observed with the RSF1010 plasmid, which has the mob gene (endonuclease-encoding) and the oriT site (nicked by mob gene product). This kind of plasmid is not self-transmissible, but is mobilizable if provided the tra function (pili) in trans. The third type of transfer is seen with the ColEl, pBR322 and pUC18 plasmids. These have the bom (basis of mobility) site. This kind of plasmid is nonconjugative, but can be mobilized if provided pili and the mob gene product (endonuclease) in trans (Freifelder 1983).

Conjugation of Rhizobium. Conjugation has been commonly utilized for the transfer of genes to Rhizobium. Pankhurst et al. (1983) demonstrated the transfer of an indigenous plasmid of Rhizobium loti to other Rhizobia and Agrobacterium tumefaciens. Beringer (1974) demonstrated R factor (RP4, RK2) transfer from E. coli to R. leguminosarum
and vice versa. Hooykaas et al. (1982) conducted phenotypic expression studies of mutations by transferring R plasmids to *R. meliloti*.

As noted above, conjugation seems to be the most suitable method of gene transfer to *Rhizobium*. For this reason, the conjugation method was commonly utilized for the genetic manipulation experiments of *Rhizobium* described in the previous section for this paper.

**Transduction of Rhizobium.** Transduction has not been commonly utilized for gene transfer to *Rhizobium*. However, there are some examples of transduction experiments using *Rhizobium* in the literature.

As an example, the generalized transduction of *R. meliloti* with bacteriophage N3 was carried out by Martin and Long (1984). Additionally, Kahn and Timblin (1984) have placed the *E. coli* lactose operon into *R. meliloti* by transduction using bacteriophage P2 and HK113C.

**Cloning vectors for Rhizobium**

The primary cloning vectors which have been used for the genetic manipulation of rhizobia are the IncP plasmids, which can replicate in a variety of Gram-negative bacteria because of their broad host range properties. These include pRK290, RP1, RP4, R68.45 and pSUP204.

pRK290 (20kb) contains a functional RK2 replicon and is non-self-transmissible, but can be mobilized at high
frequency by using a helper plasmid. pRK2013 is commonly used as the helper plasmid and consists of the RK2 transfer genes cloned onto a ColE1 replicon. By using this pRK290 and pRK2013 system, a gene bank of *R. meliloti* was constructed (Ditta et al. 1980). The cosmid cloning vector pLAFR1 (21.6kb), which has the lambda cos site in pRK290, was also constructed by Friedman et al. (1982). Plasmids R68.45 and RP4, originally isolated from *Pseudomonas*, were used for the mapping of *Rhizobium* genes (Megias et al. 1982; Scott and Ronson 1982; Meade and Signer 1977) due to their ability to carry out the conjugational transfer of chromosomal markers. pSUP204 (12kb) was developed from the RSF1010 replicon and the pBR325 antibiotic resistance genes (Am\(^\text{R}\), Cm\(^\text{R}\) and Tet\(^\text{R}\)) (Simon et al. 1983).

In addition to these vectors, pGC91.14 (RPl::Tn951) was developed for experiments involving *E. coli* and *Rhizobium* conjugation (Johnson 1985). Transposon (Tn5) mutagenesis has also been done by using a suicide plasmid vector (pACYC177; p15A replicon) which functions in *E. coli* but not in *Rhizobium* (Selvaraj and Iyer 1983). The chimeric plasmid pUW942 with the RP4 transfer genes and the ColE1 replication region, was constructed to introduce transposons Tn501 and Tn7 into *R. japonicum* (Arunakumari and Vidaver 1986). pUW942 is not maintained in *R. japonicum* as an autonomously replicating plasmid. Instead, all or part of it is either integrated into the chromosome or the resident plasmids of *R.
31

japonicum. pGS9 with pJB3JI (kanamycin sensitive derivative of R68.45), was used for Tn5 mutagenesis (Thomas et al. 1986). DF2 phage, in addition to R 68.45, was used for mapping of the R. meliloti chromosome by cotransductional analysis (Casadesus and Olivares 1979).

Some researchers have developed integration vectors using the insertional mechanisms proposed by Leenhouts et al. (1989). Williams et al. (1988) developed a genomic integration vector (pMW193; pRK290 carrying a chromosomal fragment of R. meliloti) using a genomic insertion system for R. meliloti. The integrable vector, pRJ1035 based upon the pCMB1 replicon, was also developed for the site directed, genomic integration of foreign DNA into Rhizobium (Acuna et al. 1987). Legocki et al. (1984) constructed the Rhizobium expression vector pREV1000, which consists of the nifH promoter, the RP4 oriT site (mob region), Am<sup>F</sup> and the ori region of pBR322. In addition, a non-essential chromosomal fragment of Rhizobium has been included to create an insertional vector system via this chromosomal homology.

So far, there are no cloning vectors available for the genetic manipulation of Rhizobium which are based upon a naturally occurring Rhizobium plasmids. Although efforts have been made, up to now these have been unsuccessful (Puhler et al. 1981).

Significance of Rhizobium to the environment
Rhizobia species are agriculturally very important soil bacteria due to their ability to fix atmospheric nitrogen into ammonia in nodules of leguminous plant roots. A better understanding of Rhizobium will therefore greatly contribute to advances in agriculture and related areas.

Pesticides are chemicals for the control of pests. Pesticides are categorized on the basis of the kinds of organisms on which they act. Thus, insecticides, herbicides and fungicides are designed for the control of insects, weeds and fungi, respectively. However, the usage of pesticides for increased agricultural production may have deleterious effects on nonpest, or benevolent, species of organisms like Rhizobium (Materon and Weaver 1984). Pesticide treatment has been shown to affect the growth of Rhizobium, both inoculant rhizobia and soil populations of rhizobia. This can suppress nodulation initiation and the development of legumes, resulting in actual reduced crop yields (Alexander 1977). One report states that Rhizobium sensitivity to fungicide affects the survival of Rhizobium, nodulating activity and its N₂ fixing capacity (Diatloff 1986). A similar effect was observed when soybeans were treated with a range of herbicides where again nodulation was retarded up to 50%. Many herbicides affect root development or chlorophyll synthesis and it is therefore not surprising that they influence nodule formation and function.

Insecticides have also been implicated in the death of
inoculant rhizobia. Cyclodiene insecticides (e.g., Dieldrin®, Aldrin®) have an effect on the nodulation of Phaseolus lathyroides and Vigna marina in the field.

Pesticide residues remaining in the soil can also cause environmental pollution by contaminating the ground water or by entering the food chain via accumulation and biomagnification. For example, the subtle, long term and less easily defined increase in particular types of cancer due to accidental or occupational exposure to pesticides has arisen from the use of synthetically produced pesticides.

There are some plasmids known to encode proteins capable of degrading pesticides. Plasmids pJP1 (90 kb) and pJP3 (77 kb), isolated from Alcaligenes paradoxus, can degrade the herbicide 2,4-D (dichlorophenoxyacetic acid) (Don and Pemberton 1981; Fisher et al. 1978). Plasmid pCS1 (66 kb), isolated from Pseudomonas diminuta, was found to hydrolyze parathion (Serdar et al. 1982).

By using the technology of genetic engineering, genes responsible for the detoxification or degradation of pesticides may be cloned into broad host range vectors. Novel strains may then be created by introducing these vectors into suitable hosts. These could be useful for degrading or detoxifying those toxic pesticide molecules which are real or potential threats to both man and his environment (Pemberton and Wynne 1984).

Parathion is a broad spectrum organophosphate insecticide
used to kill harmful insects. The parathion hydrolase gene, which cleaves parathion into relatively nontoxic molecules (Fig. 4), has been localized to the *Pseudomonas putida* plasmid pCMS1. This gene has been cloned into several vectors and its complete nucleotide sequence determined (Serdar and Gibson 1985; Serdar et al. 1989). In addition, three non-identical parathion hydrolases have been isolated, purified and characterized from *Flavobacterium*. They were found to be a 35 kilodalton single subunit membrane bound protein, a 43 kilodalton single subunit cytosolic protein and a membrane bound protein of 4 identical 67 kilodalton subunits, respectively (Mulbry and Karns 1989).

The parathion hydrolase gene from *P. putida* was selected in this project as a useful marker biodegradation gene for introduction into *Rhizobium*. Even though some of the naturally occurring *Rhizobium* species are known to metabolize parathion, this metabolism is at a very low level and the soil population of *Rhizobium* was still affected by the presence of $>10^{-6}$ M parathion (Mick and Dahm 1970).

Therefore, *Rhizobium* which acquired enhanced parathion biodegradation ability could break down parathion residue in the soil, contributing to its own survival and nodulation activity as well as removing a toxic pollutant from the environment.

In this paper, studies of *Rhizobium* plasmids for the construction of a *Rhizobium* cloning vector and an application
Fig. 4. Reaction catalysed by parathion hydrolase. Parathion diethyl (MW 293) was hydrolysed to diethylthiophosphoric acid and p-nitrophenol (yellow color) by parathion hydrolase (adapted from Serdar et al. 1985).
\[
\begin{align*}
\text{Parathion ethyl} & \quad \xrightarrow{\text{Parathion hydrolase}} \\
\text{Diethyl thiophosphoric acid} & + \quad \text{p-nitrophenol} \\
& \quad \text{(anion form is yellow)}
\end{align*}
\]
of the cloning vector to protect *Rhizobium* from pesticides and to solve an environmental soil pollution problem are described.
CHAPTER II

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 3. *Rhizobium meliloti* 220-13 and *E. coli* S17-1 were kindly provided by Dr. U. B. Priefer (University of Bielefeld, Germany). *Rhizobium japonicum* USDA 191 was provided by Dr. A. G. Atherly (Iowa State University). The *E. coli* HB101 strain carrying pCMS40 (a recombinant pUC9 expressing the parathion hydrolase gene) was provided by Dr. C. M. Serdar (Amgen Inc., California).

Media and culture conditions

Tryptone yeast extract (TY) media (5.0 g Bacto™-tryptone, 3.0 g yeast extract and 0.3 g CaCl₂ in 1 L H₂O), *Rhizobium* minimal media (RMM) (1.0 g K₂HPO₄, 1.0 g KH₂PO₄, 0.25 g MgSO₄·7H₂O, 0.1 g CaCl₂·6H₂O, 1.0 g NH₄Cl and 10.0 g mannitol in 1 L H₂O) or yeast mannitol (YM) media (10.0 g mannitol, 0.5 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.1 g NaCl, 0.01 g CaCO₃ and 0.5 g yeast extract in 1 L H₂O) were utilized for the culture of *R. japonicum* 191. TY media or Luria-Bertani (LB) media (10 g Bacto-tryptone, 5 g yeast
Table 3. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Characteristics*</th>
<th>Source/Reference</th>
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<tr>
<td>pRj191</td>
<td>wild type</td>
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<tr>
<td>pRm220-13</td>
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<tr>
<td>Strains/plasmids</td>
<td>Characteristics*</td>
<td>Source/Reference</td>
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<tr>
<td>pRm220-13'</td>
<td>lost 1st largest plasmid from pRm220-13</td>
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<tr>
<td>pBR325</td>
<td>pMB1 replicon, Am(^{R}), Tet(^{R}), Cm(^{R})</td>
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<tr>
<td>R68.45</td>
<td>Am(^{R}), Km(^{R}), Tet(^{R}), Cma(^{+})</td>
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<tr>
<td>pSUP202</td>
<td>pMB1 replicon, RP4Mob(^{+}), Am(^{R}), Tet(^{R}), Cm(^{R})</td>
<td>U. Priefer</td>
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<tr>
<td>pCMS40</td>
<td>pUC7 carrying 1.5 kb parathion hydrolase gene fragment</td>
<td>C. Serdar</td>
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<tr>
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<td>pSUP202 carrying 1.3 kb HindIII fragment</td>
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* Sm, streptomycin; Tp, trimethoprim; pro, proline; tra, transfer; Mob, mobilizable; Am, ampicillin; Tet, tetracycline, Cm, chloramphenicol, Km, kanamycin; Cma, chromosome mobilizing ability
extract and 10 g NaCl in 1 L H2O) were utilized for the culture of \textit{R. meliloti} 220-13. TY media were commonly utilized for the culture of other \textit{Rhizobium} strains. All cultures of \textit{Rhizobium} strains were grown at 30°C.

LB media was utilized for the culture of \textit{E. coli} strains. \textit{E. coli} strains were grown at 37°C.

All liquid cultures were incubated in a New Brunswick	extsuperscript{TM} Environmental Gyrotory Shaker at 250 rpm. For solid media (agar plates), agar was added to the final concentration of 1.5%. All media were autoclaved at 121°C for 15 minutes.

Antibiotic concentrations, when utilized, were 50 µg/ml chloramphenicol, 50 µg/ml kanamycin, 50 µg/ml ampicillin or 15 µg/ml tetracycline.

\textbf{Enzymes, buffers, oligonucleotide adaptors and radioactive isotopes}

Restriction endonucleases were from BRL (Bethesda Research Laboratories), New England Biolabs and IBI (International Biotechnologies, INC.). Carlos buffer at normal strength (33 mM Tris-acetate pH 7.9, 66 mM potassium acetate pH 7.9, 10 mM magnesium acetate, 3 mM spermidine and 0.1 mg bovine serum albumin per ml) with 1X DTT (2.5 mM) was used as the digestion buffer for all restriction endonucleases. These were made and stored separately as 10X Carlos buffer and 10X DTT.

Ligation buffer for T4 ligase reactions was made as a 5X
stock containing 250 mM Tris-HCl (pH 7.5), 50 mM MgCl$_2$, 5 mM ATP and 50 mM 2-mercaptoethanol (Perbal 1984).

Kinase buffer for T4 polynucleotide kinase reactions was made as a 10X stock containing 500 mM Tris-HCl (pH 7.6), 100 mM MgCl$_2$, 50 mM DTT, 1 mM spermidine and 1 mM EDTA (Maniatis et al. 1982).

The BamHI-PstI oligonucleotide adaptor was purchased from Biosynthesis, Inc. (Lewisville, Texas).

$\gamma$-32P ATP utilized to label the probes for hybridization experiments was purchased from ICN Biomedicals, Inc.

Electrophoretic analysis of DNA samples

Electrophoresis is a method whereby charged molecules in solution, such as nucleic acids, migrate in response to an electrical field. Their rate of migration or mobility through the electrical field depends upon the net charge, size and shape of the molecules and the ionic strength, viscosity and temperature of the medium.

**Horizontal agarose gel electrophoresis.** The equipment for electrophoresis (horizontal agarose, vertical agarose and vertical polyacrylamide) was custom-made (Dr. R. C. Benjamin at University of North Texas).

Horizontal agarose minigels (5.5 cm x 9 cm) were commonly utilized to determine the plasmid content of *E. coli* and *Rhizobium* strains. Horizontal large agarose gels (11 cm x 15
cm) were also utilized for the analysis of many samples, such as DNA samples from clones carrying recombinant plasmids or restriction endonuclease analysis of plasmids.

A 0.5% agarose gel was utilized for DNA detection and isolation above 1 kb. A 0.5% agarose gel was made by adding 0.5 g agarose to 100 ml of 1X TBE buffer (0.089 M Tris, pH 8.0, 0.089 M boric acid and 0.002 M EDTA) and boiling for 2 minutes in a microwave oven.

Glass microscope slides were also used as "microgel trays" for analyzing the DNA samples of particularly small volumes (less than 2 µl) (Fig. 5).

Horizontal agarose minigels were usually electrophoresed at 60 V (about 30 mA). Large agarose gels were also run at 60 V, giving about 50 mA of current.

**Vertical agarose gel electrophoresis.** Vertical agarose gels were utilized to separate DNA samples from which individual DNA bands were to be cut out for electroelution and recovery of specific DNA fragments.

The sizes of glass plates used for vertical agarose electrophoresis were 20 cm x 20 cm (back) and 20 cm x 22 cm (front).

Prior to assembly of vertical agarose gel cassettes, the two glass plates were cleaned with a glass cleaning solution such as Windex® and then with acetone or non-denatured 95% ethanol. Following cleaning, the glass plates were wiped with a Kimwipe® dampened with a solution of 1%
Fig. 5. Simple electrophoresis using a glass microscope slide. 1. Electrophoresis comb supported by clips. 2. Overview of glass microscope slide with well-forming comb. 3. Side view of microscope slide onto which an agarose gel has been poured (usually 4 ml).
1. Clip ↓ Comb ↓

2. Clip ← Comb

Microscope slide glass

3. Comb

Agarose gel

Microscope slide glass
dimethyldichlorosilane in heptane. The glass plates were then allowed to dry for a few minutes. Two 20 cm x 1 cm x 1.5 mm Delrin® spacers, covered with a very thin layer of silicone vacuum grease (half width of both sides), were placed along the edges of the smaller glass plate. An 18.5 cm x 1 cm x 5 mm cellulose sponge soaked in 1X TBE buffer was also placed along the bottom of the glass plate between the two spacers. The remaining glass plate was finally placed over the sponge/spacer/plate assembly and clamped along each side with 3 medium size binding clips.

The vertical agarose gel electrophoresis was performed at 80 V (about 20 mA).

Polyacrylamide gel electrophoresis. Vertical polyacrylamide gel electrophoresis was utilized for the analysis of DNA fragments less than 1 kb in size.

The treatment of glass plates for the polyacrylamide gel cassette was the same as that used for the vertical agarose gel electrophoresis cassette. The assembly of the actual gel cassette for the polyacrylamide gel electrophoresis was also similar to that for vertical agarose gel electrophoresis with one major exception. A Delrin® spacer was placed at the bottom of the cassette during gel pouring instead of the sponge used with agarose cassettes.

A 6% polyacrylamide gel solution was routinely used. This gel solution was made by mixing 12 ml of 40% acrylamide/bisacrylamide stock solution (40 g acrylamide and
1 g bisacrylamide in 100 ml H₂O), 16 ml of 5X TBE (445 mM Tris, pH 8.3, 445 mM boric acid and 10 mM EDTA), 100 mg solid ammonium persulfate (\(\text{(NH}_4\text{)}_2\text{S}_2\text{O}_8\)) and 52 ml of H₂O. The gel solution was de-gassed in vacuo until all dissolved gases were removed. Then, 40 µl of TEMED was added and the solution swirled rapidly. The gel solution was poured into an assembled gel cassette, taking care not to introduce air bubbles. The polymerized polyacrylamide gel was pre-electrophoresed for 1 hour at 300 V (about 30 mA). 5X TBE buffer was made as a stock solution and used for both agarose and polyacrylamide gels. A 5X TBE buffer was diluted to 0.5X TBE for agarose gels and 1X TBE for polyacrylamide gels (Sambrook et al. 1989). A 5X loading buffer (0.4% bromophenol blue, 0.4% xylene cyanol, 25% glycerol, 5X TBE) was also prepared as a stock solution for agarose and polyacrylamide gel electrophoresis.

The polyacrylamide gel electrophoresis was carried out at 250 V (about 20 mA).

**Observation of DNA bands.** The gels were stained in Ethidium Bromide staining solution (0.5 µg/ml) following electrophoresis. Occasionally, EtBr was added to the 5X loading buffer to a final concentration 1 mg/ml. These gels could be immediately checked upon completion, or for agarose gels only, quickly monitored for band separation with a hand held UV light during the actual running of the gel.

UV shadowing (Clarke et al. 1982) was also utilized to
avoid DNA damage by EtBr when DNA was to be recovered from gels. This was possible only when large amounts of DNA were loaded onto the gel. The procedure for the detection of DNA by UV shadowing was as follows. The gel was transferred from the glass plate to a piece of plastic wrap. The gel was then placed on a thin layer chromatography (TLC) plate impregnated with an ultraviolet fluorescent indicator. The gel was illuminated from above by a short wave (about 260 nm) ultraviolet lamp. Ultraviolet radiation caused the fluorescent indicator to fluoresce visible light, giving the plate a bright glow. Any DNA in the gel will absorb the ultraviolet radiation before it strikes the plate, leaving dark bands at those locations.

Detection and isolation of plasmids

Detection of *Rhizobium* plasmids. The in-gel lysis method (Eckhardt 1978) and the method of Kado and Liu (1981) were utilized for quick detection of *Rhizobium* plasmids. These methods gave acceptable recoveries for *R. meliloti* 220-13, which has small plasmids, but worked poorly for other *Rhizobium* strains which have much larger, small copy number plasmids.

A. Modified Eckhardt (1978) in-gel lysis method. Rhizobia strains were grown in TY media to an absorbance at 550 nm of 0.2-0.3 (use only fresh cultures). A 200 µl volume of the culture was transferred to a 1.5 ml microcentrifuge
tube. The microcentrifuge tube was centrifuged at 10,000 xg for 1 minute. The bacterial cell pellet was suspended in 20 μl of a solution consisting of 25% sucrose in 1X TBE buffer, 100 μg RNase per ml and 1 mg lysozyme per ml. The suspension was transferred immediately into the well (length 6 mm, width 1.5 mm, height 6 mm) of an agarose gel containing 0.2% SDS. The samples were left undisturbed in the well for 10 minutes during the lysozyme treatment. The gel was then electrophoresed for 30 minutes at 20 volts (about 10 mA) to allow the "SDS treatment" to occur. The gel was subsequently electrophoresed a further 2-3 hours at 100 volts (about 40 mA).

B. Modified Kado and Liu (1981) method. One ml of bacterial culture was placed in a 1.5 ml microcentrifuge tube and centrifuged at 10,000 xg for 1 minute. The bacterial cell pellet was suspended in 50 μl of a lysing solution containing 50 mM Tris (pH 12.6) and 3% SDS. The cell suspension was then incubated at 60°C for 30 minutes. The cell lysate was extracted with an equal volume of phenol/chloroform mixture (1:1). This mixture was then centrifuged at 10,000 xg for 10 minutes. A 30 μl volume was taken from upper phase of the tube and loaded onto an agarose gel for electrophoretic analysis.

Large scale isolation of Rhizobium plasmids. In order to carry out large scale (100 ml-1 L cultures) isolations of Rhizobium plasmids, the Schwinghamer (1980) method and the
Johnston and Gunsalus (1977) method were utilized. In this study, the Schwinghamer method was more frequently utilized for the isolation of the large Rhizobium plasmids because of its relatively simple protocol.

A. Modified Schwinghamer (1980) method. One hundred ml of bacterial cell culture was centrifuged at 7,000 xg for 10 minutes. The cell pellet was resuspended in 5 ml of TEN (50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 50 mM NaCl) containing 0.1% sarkosyl (sarkosyl washing facilitates subsequent cell lysis). The cell suspension was centrifuged again at 7,000 xg for 10 minutes. Then, 2 ml of TEN solution was added to the cell pellet and the tube vortexed well. Two ml of sucrose mix solution (30% sucrose, 500 mM Tris-HCl, pH 8.0 and 100 mM EDTA) was then added to the cell suspension and mixed by inversion. This was incubated at 0°C for 10 minutes. One ml of lysozyme solution (10 mg/ml in 250 mM Tris-HCl, pH 8.0) was added and the incubation continued at 0°C for 10 minutes. Three ml of 250 mM EDTA was added to the mixture, which was incubated at 0°C for a further 10 minutes. Two ml of 25% sarkosyl/5% deoxycholic acid mixture was added to the samples, which were incubated at 0°C for 1 more hour. The cell lysate was then centrifuged at 80,900 xg for 1 hour (clearing spin). Eight ml of supernatant was removed and 8.40 g CsCl and a 200 μl of EtBr (20 mg/ml) were added to this supernatant. Then, this mixture was centrifuged at 118,700 xg (T1270 rotor from DuPont) for 40 hours.
B. Modified Johnston and Gunsalus (1977) method. One liter of bacterial culture was centrifuged at 8,000 xg for 10 minutes. A 100 ml volume of 50 mM Tris-HCl, pH 8.0 and 25% sucrose was added to the bacterial cell pellet and vortexed. Then, 20 ml of lysozyme solution (10 mg/ml in 250 mM Tris-HCl, pH 8.0) was added to the cell suspension and mixed by inversion. This mixture was incubated at 0°C for 10 minutes. A 20 ml volume of 250 mM EDTA was added to the cell suspension which was further incubated at 0°C for 10 minutes. Next, 50 ml of 5 M NaCl was added to the cell suspension, followed by a 40 ml volume of 25% sarkosyl/5% deoxycholic acid. These were mixed well and incubated at 0°C for 1 hour. This lysate was centrifuged at 80,900 xg (T647.5 rotor) for 1 hour. A 0.25 volume quantity of 50% PEG 8,000 was added per 200 ml of supernatant. This mixture was incubated at 4°C overnight and centrifuged at 16,000 xg for 10 minutes. The PEG pellet was resuspended in 16 ml of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). Each 16 ml sample was mixed with 16.8 g CsCl and 400 μl of EtBr (20 mg/ml). These were thoroughly mixed, sealed in tubes and centrifuged at 118,700 xg (T1270) for 40 hours.

Isolation of E. coli plasmids. The alkaline lysis method (Maniatis et al. 1982) was utilized for small scale (5 ml) E. coli plasmid isolations. The Tanaka and Weisblum (1975) method and the Timmis et al. (1978) method were utilized for preparative scale (1 L) E. coli plasmid isolations.
A. Modified alkaline lysis method. A 1.5 ml volume of bacterial cell culture was centrifuged at 10,000 xg for 1 minute. The cell pellet was resuspended by vortexing with 100 μl of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0) and 4 mg lysozyme per ml. This cell suspension was lysed by adding 200 μl of 0.2 N NaOH containing 1% SDS and mixing by inversion. The cell lysate was incubated on ice for 5 minutes. A 150 μl volume of potassium acetate solution (made by mixing 6 ml of 5 M potassium acetate, 1.15 ml of glacial acetic acid and 2.85 ml of H2O) was added to the cell lysate and vortexed briefly. The lysate was incubated on ice for a further 5 minutes. An equal volume of phenol/chloroform mixture (1:1) was added to the cell lysate and mixed by inverting several times. This lysate was centrifuged at 10,000 xg for 10 minutes. The upper layer (aqueous) was taken from the tube and transferred to a new microcentrifuge tube. Two volumes of ethanol were added and the contents mixed completely. The precipitate was collected by centrifugation at 10,000 xg for 10 minutes. The final plasmid pellet was redissolved in 50 μl of TE buffer.

B. Modified Tanaka and Weisblum (1975) method. A 50 ml volume of LB broth was inoculated with the desired strain and incubated overnight in a New Brunswick™ gyratory shaker at 37°C (250 rpm). A 10 ml volume from the 50 ml overnight culture was then transferred to 1 L of LB broth in a 2.8 L Fernbach® flask. This culture was further incubated at 37°C.
(250 rpm) until the absorbance at 550 nm reached about 0.8-1.0 (late log phase growth). Then 2 ml of 85 mg chloramphenicol per ml dissolved in 95% ethanol was added (final concentration, 170 µg/ml). The culture was incubated overnight at 37°C on the shaker at 250 rpm. The cells were collected in a GSA rotor (RC5C, Sorvall® Instruments) at 8,000 xg for 10 minutes. The cell pellet was resuspended in 20 ml of 0.15 M NaCl and transferred to one 45 ml Oak Ridge style centrifuge tube. The cell suspension was centrifuged in an SA600 rotor (RC5C, Sorvall® Instruments) at 7,000 xg for 10 minutes at 4°C. The pellet was resuspended in 10 ml of 50 mM Tris-Cl pH 8.0/25% sucrose. The cell suspension was mixed with 2 ml of lysozyme solution (5 mg/ml) by inversion. The sample was kept on ice for 5 minutes and 4 ml of 0.25 M Na₂ EDTA (pH 8.0) added. This was mixed by inversion and left on ice for a further 5 minutes. A 5 ml volume of 5 M NaCl was then added and mixed quickly by inversion. Two ml of 10% SDS was added, mixed by inversion and the resulting suspension was left on ice in the cold room for 2 hours. This cell lysate was then centrifuged at 40,000 xg for 60 minutes in an SA600 rotor. The supernatant was poured off into a graduated cylinder and 1 volume of isopropanol was added. The mixture was transferred into a GSA bottle and placed in a dry ice/ethanol bath for 20 minutes (or overnight in a -20°C freezer). The contents were melted by placing the tube in tap water for a short time. The sample was
centrifuged in a GSA rotor at 16,000 xg for 10 minutes. The resulting pellet was resuspended in 8 ml of TE buffer. The suspension was placed in a cold room on a magnetic stirrer with a stirring bar inside the GSA bottle. Heat-treated RNase was added to a final concentration of 20 μg/ml to destroy any tRNA present. The suspension was centrifuged at 16,000 xg for 10 minutes. This final crude plasmid preparation was then ready for ultracentrifuge purification.

An EtBr/CsCl gradient was utilized for further purification of large scale plasmid preparations. An amount of solid CsCl equivalent to 1.05 g of solid CsCl per 1 ml of supernatant was added to the plasmid solution. The CsCl plasmid solution was transferred to an ultracentrifuge tube (for T1270 rotor) by Pasteur pipet. Two hundred μl of 20 mg/ml EtBr was added per ultracentrifuge tube. Any remaining space in the top of the ultracentrifuge tube was filled with balancing solution (1.05 g CsCl/ml TE buffer). The tubes should be balanced with each other within a 0.02 g deviation. The tubes were sealed with Ultracrimp™ caps using a Dupont crimping device. The tubes were inverted several times to mix the EtBr and the plasmid solution before loading into the ultracentrifuge. The mixture was centrifuged for 40 hours at 118,700 xg (T1270 rotor, OTD75B Sorvall® ultracentrifuge). The lower band was removed (Fig. 6) using a 20 gauge hypodermic needle inserted through the side of the tube. The EtBr was extracted from the plasmid solution using butanol
Fig. 6. Diagramatic representation of a plasmid DNA band in an EtBr/CsCl-containing ultracentrifuge tube. The plasmid (covalently closed circular) band is always located below the chromosomal band in the EtBr/CsCl gradient due to its higher density.
20 Gauge needle

- Protein
- Chromosomal DNA
- Plasmid DNA (Covalently closed circular)
- RNA
saturated with TE buffer. A 2 volume quantity of H₂O and 9 volumes of ethanol were then added to the EtBr-free plasmid solution. The mixture was placed at -70°C for 10 minutes and centrifuged at 14,500 xg for 10 minutes. The plasmid pellet was resuspended in 500 μl of TE buffer. The solution was transferred to a 1.5 ml microcentrifuge tube and 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol were added. The mixed solution was centrifuged at 10,000 xg for 10 minutes. The pure plasmid pellet was resuspended in 500 μl of TE buffer and stored at -20°C.

C. Modified Timmis (1978) method. One liter of bacterial cell culture was harvested by centrifugation at 8,000 xg for 10 minutes. The cell pellet was resuspended in 12 ml of 25% sucrose solution containing 0.05 M Tris-HCl, pH 8.0. A 2 ml volume of lysozyme solution (5 mg/ml in 0.25 M Tris-HCl) was added to this cell suspension. Following mixing, 4 ml of 0.25 M EDTA was added and the mixture incubated at room temperature for 5 minutes. At this time, 15 ml of triton detergent solution (2% triton X-100 in 0.05 M Tris-HCl, pH 8.0) was added and the cell lysate incubated at 0°C for 15 minutes. The cell lysate was then centrifuged at 40,000 xg for 15 minutes. An 8 ml volume of cleared lysate was mixed with 8 g CsCl plus 1 ml of EtBr (5 mg/ml in H₂O) and sealed in an ultracentrifuge tube. This was centrifuged at 118,700 xg (T1270 rotor) for 40 hours. Plasmid recovery from tube was carried out as described
above.

**Curing of R. meliloti 220-13 plasmids**

High temperature (37°C) treatment was used for curing *Rhizobium meliloti* 220-13 plasmids (Higashi et al. 1983; Morrison et al. 1983). *R. meliloti* 220-13 was streaked onto fresh LB agar plates. Streaked agar plates were incubated at 37°C for one week. A small number of colonies appeared on these plates (generally less than 10 colonies per plate). Colonies which survived during heat treatment were cultured in 5 ml of LB medium at 30°C for one day. Plasmid isolations were performed on the cultures and these preparations were compared to the complement of plasmids from the original strain.

**Conjugation and electroporation of Rhizobium strains**

*Conjugation of Rhizobium.* A simple plate mating technique was utilized for the conjugation of *E. coli* and *Rhizobium* strains. The *E. coli* strain was grown in 5 ml of LB media at 37°C for one day. The *Rhizobium* strain was grown in 5 ml of LB media (in the case of *R. meliloti*) or TY media (other *Rhizobium* strains) at 30°C for one day. A 100 µl volume of each culture was mixed and spread on a non-selective LB plate or TY plate. The plate was incubated overnight at 30°C. The bacterial growth on this plate was then carefully restreaked onto selective media. This
restreaking was repeated several times until *Rhizobium* colonies were distinctively separated from *E. coli* colonies on the selective plate.

**Electroporation of *Rhizobium***. Electroporation was conducted according to Guerinot et al. (1990) using a Biorad Gene Pulser® electroporation apparatus. *Rhizobium* strains were cultured in 5 ml of TY or LB media for one day at 30°C. The entire cell culture was collected in a 1.5 ml sterile microcentrifuge tube by centrifuging at 3,000 xg for 1 minute two or three times. The cell pellet was washed with 1 ml of sterilized distilled H2O twice to remove any residual media. Cells were collected each time by centrifugation and resuspended by gentle vortexing. The final cell pellet was resuspended in 400 μl of H2O. Twenty μl of plasmid (0.1-0.5 μg/μl) was then added to the cell suspension. The mixture of cell suspension and plasmid was transferred to the electroporation cuvette (0.2 cm electrode gap, disposable, prechilled to -20°C). The cuvette was electroporated at 800 ohms, 25 μF and 2.5 KV (time constant 3.0-5.0 msec). A 0.5 ml volume of LB media or TY media was added to the cuvette immediately after the pulse treatment. The cuvette was incubated at 30°C for 1 hour and the entire contents plated on selective media.

**Transformation of *E. coli* strains**

The microcentrifuge tube (1.5 ml Effendorf™ tube)
transformation technique developed by the author (PS Jeong) during this project was utilized for the transformation of *E. coli*. An *E. coli* culture was grown in 5 ml of LB media at 37°C overnight. A 1.5 ml volume of culture was transferred into a microcentrifuge tube and the cells collected by centrifuging at 3,000 xg for 1 minute. The cell pellet was washed with 1 ml of 10 mM Tris-HCl, pH 8.0 and 10 mM NaCl. This cell suspension was again centrifuged at 3,000 xg for 1 minute. The cell pellet was resuspended in 1 ml of 50 mM CaCl₂ and 10 mM Tris-HCl, pH 8.0. This cell suspension was incubated for 30 minutes on ice. Following this incubation, the cells were collected by centrifugation at 3,000 xg for 1 minute. The cell pellet was resuspended in 200 μl of 50 mM CaCl₂ and 10 mM Tris-HCl, pH 8.0. A 20 μl volume of the plasmid solution, whose concentration ranged from 0.1-0.5 μg/μl, was added to the cell suspension. This was then incubated on ice for 30 minutes. This cell mixture was heat shocked at 42°C for 1 minute. Next, 300 μl of LB medium was added and the cell mixture was incubated in the microcentrifuge tube for 1 hour at 37°C without shaking. A 200 μl volume of the cell mixture was then spread per selective plate.

**DNA fragment elution from agarose gels**

Small scale DNA fragment purification from agarose gels. The Gene Clean™ (Bio 101 Inc.) method was utilized for the
isolation of small quantities (<2 μg) of DNA fragments from agarose gels. An agarose gel slice which contained the DNA fragment of interest was cut from the gel. Three volumes of NaI solution (provided by the manufacturer) were added to the gel slice in a 1.5 ml microcentrifuge tube. This microcentrifuge tube was incubated at 55°C for 10 minutes to dissolve the gel slice. One μl of silica suspension (extremely finely ground glass) was added per μg of DNA in the gel slice. This mixture was incubated at room temperature for 5 minutes and then centrifuged at 10,000 xg for 5 seconds. The supernatant was discarded. The silica pellet was washed with 1 ml of EtOH mixture (solution provided by the manufacturer) by gentle pipeting and centrifuging at 10,000 xg for 5 seconds. This was then repeated. The final supernatant was again discarded. One μl of H2O was then added per 1 μl of silica utilized. The silica suspension was incubated at 50°C for 5 minutes in order to elute the DNA. The silica was removed from the eluted DNA by centrifuging at 10,000 xg for 30 seconds. The eluted DNA was transferred to a new microcentrifuge tube and stored at -20°C.

Preparative scale DNA fragment purification by electrophoresis.

A. Preparation of dialysis tubing. Dialysis tubing (Spectrapor®, MW cutoff 6,000-8,000, flat width 23 mm) was utilized for the isolation of preparative scale amounts of
DNA from agarose gels. The dialysis tubing was cut to proper length (about 8 cm) and boiled in 2% NaHCO₃ and 1 mM EDTA solution for 10 minutes. The dialysis tubing was then washed with distilled H₂O thoroughly and boiled again in H₂O for 10 minutes. The dialysis tubes were allowed to cool and stored at 4°C in 0.1% sodium azide solution.

B. Electroelution. The agarose gel slice containing the DNA fragment of interest was placed inside a dialysis tube clipped at one end with a dialysis bag clip. A 500 μl volume of 0.25X TBE buffer was added to the dialysis bag and the other end of the tubing sealed with a second dialysis bag clip. The dialysis tubing was then placed into a horizontal agarose gel electrophoresis unit which had been filled with 0.25X TBE buffer. The level of buffer was adjusted until it just covered the dialysis tubing. The DNA was electroeluted for 2 hours at 80 V (about 30 mA). Upon completion of electroelution, reverse current was applied for 15 seconds. One of the clips at the end of the dialysis tubing was taken off and the buffer inside the tubing was transferred to a 1.5 ml microcentrifuge tube using a pipet tip. An equal volume of phenol/chloroform mixture was added to the eluate and vortexed well to remove contaminating agarose debris. The mixture was then centrifuged at 10,000 xg for 10 minutes. The upper layer (aqueous phase) was transferred to a new microcentrifuge tube and 0.1 volume of 3 M sodium acetate plus 2 volumes of absolute EtOH were added. This was then
mixed well and centrifuged at 10,000 xg for 10 minutes. The DNA pellet was dissolved in 20 µl of TE buffer and stored at -20°C.

**Radioactive labelling of DNA probes**

DNA fragments isolated from gel slices were treated with BAP (bacterial alkaline phosphatase) at 65°C for 3 hours to remove phosphate groups from the 5' ends. An equal volume of phenol/chloroform mixture was then added and vortexed well to terminate the reaction. The mixed solution was centrifuged at 10,000 xg for 10 minutes to resolve the organic and aqueous phases. The upper layer (aqueous) was transferred to a new microcentrifuge tube and 0.1 volume of 3 M sodium acetate plus 2 volumes of ethanol were added and mixed completely. The microcentrifuge tube was centrifuged at 10,000 xg for 10 minutes. The dephosphorylated DNA was dissolved in 20 µl of TE buffer. This DNA was then radioactively labeled using T4 polynucleotide kinase (20 units), 10X kinase buffer (10 µl), gamma-\(^{32}\)P ATP (usually 150 µCi) and water up to 100 µl of reaction volume. The kinase mixture was incubated at 37°C for 1 hour. The radioactively labeled DNA fragment was separated from unincorporated gamma-\(^{32}\)P ATP using a 1 ml syringe filled with Sephadex™ G-50 by the following procedure. The bottom of a 1 ml disposable syringe was plugged with a small amount of sterile siliconized glass wool. In the syringe, a column of
Sephadex™ G-50 equilibrated in TE (pH 8.0) containing 0.1 M NaCl was prepared. The syringe was centrifuged at 1,600 xg (H-1000B rotor, T6000B model centrifuge, Sorvall of Du Pont) for 5 minutes. The DNA sample was applied to the top of the column in a total volume of 0.1 ml. This syringe was centrifuged at exactly the same speed and for exactly the same time as before. The 100 μl volume of effluent from the syringe was collected in a decapped 1.5 ml microcentrifuge tube. The unincorporated gamma-\(^{32}\)P ATP remained in the syringe, which was carefully discarded.

**Southern blotting and hybridization**

DNA in agarose gels was transferred to nylon membranes by the Southern blotting method (Maniatis et al. 1982). The gel was placed in 200 ml of 1.5 M NaCl and 0.5 M NaOH (denaturing solution) in a dish and allowed to soak for 30 minutes. This solution was discarded and the gel was then soaked in 200 ml of 0.5 M Tris, pH 8.0 and 1.5 M NaCl (neutralizing solution) for 30 minutes. This solution was then drained and the gel rinsed with 200 ml of water. The gel was finally soaked in 200 ml of 10X SSC (1.5 M NaCl and 0.15 M sodium citrate) for 30 minutes. A pad of 3-4 sheets of Whatman 3MM paper soaked with 10X SSC was placed on Saran Wrap spread on the bench. The gel was placed on the top of the SSC-saturated paper and overlaid with a sheet of hybridization transfer membrane (Gene Screen Plus™). A 10X SSC saturated sheet of 3MM paper
was then placed on the top of the hybridization membrane and the stack was overlaid with blotting paper. A heavy object was placed on the stack. The transfer was allowed to continue overnight (about 20 hours). The hybridization membrane was carefully peeled from the gel and placed in 10 ml of prehybridization solution (1% SDS, 1 M NaCl, 10% dextran sulfate and 50% deionized formamide) in a sealable plastic hybridization bag. This bag was incubated with constant agitation for 30 minutes at 42°C. One corner of the bag was then carefully cut and 1 ml of hybridization solution containing the probe (heated to 100°C for 10 minutes for denaturation of the DNA probe immediately prior to use) was added. The plastic bag was resealed with the heat sealer and incubated at 42°C with constant agitation for 24 hours. The membrane was removed from the hybridization solution and washed twice with 100 ml of 2X SSC at room temperature for 5 minutes with constant agitation. This was followed by 2 washes at 65°C with 200 ml of 2X SSC containing 1% SDS. These were carried out with constant agitation for 30 minutes each. The final 2 washes were carried out with 100 ml of 0.1X SSC at room temperature for 30 minutes. The membrane was dried and exposed to kodak XAR-5 film using an intensifying screen at -80°C.

**Autoradiography**

Kodak X-Omat XAR-5 film was used for autoradiography.
The processing of X-ray film was done according to the following procedure. The X-ray films were developed in a tray containing developer (Kodak GBX) for 5 minutes with no agitation. The X-ray films were then transferred to another tray containing water (for rinsing) for 30 seconds with continuous agitation. The X-ray films were transferred from the water to a third tray containing fixer (Kodak GBX or Kodak RPX-Omat) for 5 minutes with intermittent agitation. Finally, the fixed films were washed with running water for 10 minutes and air dried.

Gel Photography

A Polaroid MP-4 Land Camera system was utilized for photographing DNA bands in gels. A Fotodyne U.V. transilluminator (310 nm) was utilized to illuminate the ethidium bromide-stained DNA bands. A Polaroid film Type 55 positive/negative (ISO 50) was used with this system. An F-stop of 4.5 and an exposure time of 1 minute were commonly used. However, exposure time was often adjusted due to DNA band intensities. The film was allowed to develop for 15 seconds inside the envelope after removal from camera. The film envelope was then carefully torn apart and the positive photograph peeled back. The photograph was coated with the fixer/hardner and the negative kept for future use after being soaked in 18% sodium sulfite (Na$_2$SO$_3$) for 5 minutes, washing with water and air drying.
Assay of parathion hydrolase activity in *R. meliloti* 220-13 carrying pPSJ123-PH

Several colonies were picked by toothpick and transferred to a glass tube (10 x 75 mm). A 20 μl volume of 100 mM ethyl parathion was added to the glass tube. Then 160 μl of H₂O and 20 μl of 100 mM Tris-HCl, pH 8.5 were added. The total reaction volume was thus 200 μl and the final concentration of ethyl parathion was 10 mM. The appearance of yellow color was the result of p-nitrophenol production.

Nodulation of seeds with rhizobia

The seeds (alfalfa or soybean) were sterilized with absolute EtOH for 2 minutes. These seeds were rinsed three times with sterilized H₂O. The seeds inoculated with cultures of *Rhizobium* were placed into vermiculite which had been autoclaved at 121°C for 15 minutes. The seeds were germinated and allowed to grow in a plant growth chamber under the following conditions: light period, 15 hours a day; temperature, 23°C; intensity of illumination, 20 watt fluorescent bulb x 4 (total 80 watts).
CHAPTER III

RESULTS AND DISCUSSION

Detection and isolation of *Rhizobium* plasmids

Plasmids in rhizobia are often quite large and are commonly referred to as megaplasmids. For this reason, *Rhizobium* plasmids are somewhat difficult to detect or isolate by the conventional methods developed primarily for the study of smaller *E. coli* plasmids. There are several published methods which have been utilized for the detection or isolation of *Rhizobium* plasmids. These include Eckhardt (1978), Hansen and Olson (1978), Hirsh et al. (1980), Humphreys et al. (1975), Meyers et al. (1976) and Prakash et al. (1980). Not all of these methods proved to be useful for the *Rhizobium* strains studied in this paper. A modified in-gel lysis method (Eckhardt 1978) was applied to detect plasmids in several *Rhizobium* species, but because of frequently faint and smearing bands on the gels, plasmid identification was often uncertain. However, *R. japonicum* 191 plasmids (pRj191) were successfully detected using the Casse method (Casse et al. 1979) (Fig. 7) and several plasmids were detected in *R. meliloti* 220-13 by using the alkaline lysis method (Fig. 8). For small scale (5 ml) isolations of *R. meliloti* 220-13 plasmids (pRm220-13), the
Fig. 7. Plasmids of *Rhizobium japonicum* 191. Several different preparations of pRj191 from *Rhizobium japonicum* 191. A: pRj191 digested with *Pst*I (lane 1), pRj191 control (lane 2). B: pRj191 digested with *Pst*I (lane 1), pRj191 control (lane 2). C: pRj191 digested with *Pst*I (lane 1), pRj191 control (lane 2). [Note: pRj191 refers to all of the plasmids present in this strain as a group and not a specific plasmid].
Fig. 8. Electrophoretic analysis of plasmids isolated from *Rhizobium meliloti* 220-13. Sizes of resident plasmids from *Rhizobium meliloti* 220-13 were estimated by comparison of their electrophoretic mobilities to those of plasmids of known sizes. *Rhizobium meliloti* 220-13 plasmids (lane 1), pRK290 (20 kb) (lane 2), pSUP204 (12 kb) (lane 3), pSUP202 (8 kb) (lane 4), pBR325 (6 kb) (lane 5), pBR322 (4.3 kb) (lane 6).
alkaline lysis method was commonly used. However, the alkaline lysis method did not efficiently isolate large plasmids and failed to remove much protein and chromosomal debris from some Rhizobium strains. For example, *R. japonicum* 191 plasmids were not isolated reproducibly by the alkaline lysis method. Plasmid yields and patterns from a given strain varied significantly from one isolation to the next. This was apparently due to the particularly large plasmids in this strain (>100 kb) and the low plasmid copy numbers. The Johnston and Gunsalus method (1977) and the Schwinghamer method (1980) worked well for the large scale (1 L cultures) isolation of *R. japonicum* 191 plasmids. The Schwinghamer method in particular proved to be better for the isolation of large Rhizobium plasmids because of its simple protocol.

It was also observed that some plasmids were lost during the successive subcultures of *R. meliloti* 220-13 (Fig. 9). This was presumed to be due to plasmid instability or rearrangement in Rhizobium strains (Berry and Atherly 1984; Brom et al. 1991; Djordjevic et al. 1982). This could also partially explain plasmid pattern variation from one culture to another.

*R. japonicum* 191 grew very well in RMM liquid media and produced large white, slimmy colonies on RMM agar media. However, *R. japonicum* 191 could not grow in LB media. *R. meliloti* 220-13 grew very well in LB media, but very poorly
Fig. 9. Agarose gel electrophoresis banding patterns of plasmid preparations isolated from *Rhizobium meliloti* 220-13 following successive subculturing. Plasmids (pRm220-13) isolated from a culture of *Rhizobium meliloti* 220-13 (lane 1) and plasmids (pRm220-13') from the culture of *Rhizobium meliloti* 220-13' (lane 2) which was subcultured for approximately one year. Electrophoresis conditions were 0.5X TBE buffer, 20 mA (about 90 V), 1.5 hours and a 0.5% agarose gel.
in RMM media. Therefore, RMM media was determined to be most suitable for the isolation of *R. japonicum* 191 plasmids and LB media for *R. meliloti* 220-13 plasmids.

**Cloning of *R. japonicum* 191 plasmids (pRj191) ori region into pBR325 using the pBR325/R68.45 system**

Figure 10 shows the initial strategy for the isolation of an *R. japonicum* 191 plasmid ori region using the pBR325/R68.45 system. *R. japonicum* 191 was chosen as the first *Rhizobium* strain from which to isolate a *Rhizobium* plasmid ori region to be utilized as the basis for the construction of a *Rhizobium* cloning vector. Several restriction enzymes were evaluated to determine the fragment patterns produced from *R. japonicum* 191 plasmids upon digestion (Fig. 11). Among these restriction enzymes, *PstI* was chosen for its ability to generate several suitably sized fragments from the *R. japonicum* 191 plasmids. This enzyme was also chosen because the *E. coli* vector pBR325 has only one *PstI* cleavage site, located within the Am<sup>R</sup> gene. This allows recombinant plasmids to be screened by insertional inactivation. pRj191 was digested with *PstI* and ligated into pBR325 cut with *PstI* at room temperature (23°C) for 6 hours. This ligation mixture was then transformed into *E. coli* HB101. Figure 12 shows an electrophoretic analysis of rapid isolates from HB101 transformants of some recombinant pBR325 plasmids carrying pRj191 fragments. This recombinant plasmid
Fig. 10. Schematic representation of the isolation of an ori fragment from *Rhizobium japonicum* 191 plasmids using the pBR325/R68.45 system. The ligated mixture of pBR325 digested with *Pst*I and pRj191 digested with *Pst*I was transformed into *E. coli* HB101 carrying R68.45. HB101 transformants carrying recombinant pBR325-pRj191 molecules and R68.45 were then conjugated with *Rhizobium*. The selection was made for *Rhizobium* conjugants carrying pBR325-pRj191 by Tet$^\text{r}$ or Cm$^\text{r}$ (adapted from Puhler and Timmis'1984). [Note: pRj191 refers to all plasmids in this strain].
PstI

pBR325

ligation

pRj191

pBR325/PstI

pRj191/PstI

transformation

E. coli HB101 (R68.45)

pBR325-pRj191

Rhizobium

pBR325-pRj191

R68.45

IS21

pBR325-pRj191

R68.45

cointegration

E. coli HB101 (pBR325-pRj191-R68.45)

conjugation

Rhizobium

pBR325-pRj191

R68.45

Rhizobium (pBR325-pRj191, R68.45)

if RecA⁺
Fig. 11. Agarose gel electrophoretic analysis of DNA fragments produced by restriction endonuclease cleavage of *Rhizobium japonicum* 191 plasmids. pRj191 control (lane 1), pRj191 DNA digested with *AluI* (lane 2), *CfoI* (lane 3), *EcoRV* (lane 4), *HaeIII* (lane 5), *HindIII* (lane 6), *SaiI* (lane 7), *EcoRI* (lane 8) and *PstI* (lane 9). [Note: pRj191 defined as in Fig. 10].
Fig. 12. Agarose gel electrophoretic analysis of various pBR325-pRj191 recombinant plasmids. pRj191 was digested with PstI and ligated into the E. coli vector pBR325. This ligation mix was transformed into E. coli HB101. Plasmids were isolated from transformants and analysed by 1.0% agarose gel electrophoresis. pBR325 control (lane 1), pBR325-pRj191 recombinant plasmids (lane 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15).
pBR325-pRj191 recombinants
library was used to transform an HB101 strain which possessed R68.45. The HB101 transformants carrying pBR325-pRj191 and R68.45 was selected by Cm$^R$ and Km$^R$. Some of the resulting HB101 transformants were presumed to have a cointegrated recombinant plasmid pBR325-pRj191-R68.45 which could be readily created as a result of a recombination event at the IS21 site of R68.45. These HB101 transformants were then conjugated with R. leguminosarum 10004, R. japonicum 191 and R. meliloti 10311. Among these R. japonicum 191 and R. meliloti 10311 conjugants were obtained by plating cultures onto Rhizobium minimal media (RMM) with Cm$^R$. Under these conditions, the antibiotic (Cm) kills non-transconjugant rhizobia and E. coli HB101 (pro$^-$) cannot grow on RMM. 

Rhizobium conjugants were checked for their plasmid contents. Figure 13 (panel A; lanes 5, 6 and 7) shows that two large plasmids appeared above the chromosomal band in rapid isolates from these conjugants. According to Puhler and Timmis (1984), cointegrated plasmids would be resolved into two plasmids (R68.45 and pBR325-pRj191) if the Rhizobium host is recA+. Therefore, it was tentatively presumed that one was R68.45 and the other was a recombinant pBR325-pRj191. A surviving recombinant pBR325 carried by a Rhizobium strain must have an ori region from pRj191 because the ori region of pBR325 does not function in Rhizobium strains.

Identification of recombinant pBR325 plasmids in
Fig. 13. Identification of recombinant pBR325-pRj191 plasmids, isolated from *Rhizobium* conjugants. Plasmids were isolated from *Rhizobium* conjugants carrying pBR325-pRj191 and separated by 0.7% agarose gel electrophoresis. These plasmids were transferred to nylon membranes by the method of Southern (1975) and hybridized with $^{32}$P-labeled pBR325 as a probe. A (Ethidium bromide stained gel): recombinant plasmid (pBR325-pRj191') isolated from *E. coli* HB101-1 (lane 1). HB101-1 was created by transformation of the HB101 strain with the plasmids isolated from *Rhizobium japonicum* 191 conjugant, pRj191 (lane 2), pBR325 (lane 3), R68.45 (lane 4), plasmids from *Rhizobium meliloti* 10311 conjugant (lane 5) and plasmids isolated from *Rhizobium japonicum* 191 conjugants (lane 6 and 7). B: An autoradiogram of gel following Southern blotting and hybridization is shown in panel B. [Note: pBR325-pRj191 refers to a family of recombinant plasmids constructed from pBR325 and pieces of resident plasmids. It does not refer to a single plasmid species].
A
Ethidium Bromide Stain
pBR325-pRj191
isolates

B
Autoradiogram
Rhizobium conjugants

In order to identify which, if any, plasmids in the Rhizobium conjugants were pBR325-pRj191 recombinant plasmids, hybridization of Southern blotted rapid isolates was conducted using an end-labeled HaeIII digest of pBR325 as a radioactive probe. Figure 13 (panel B; lanes 5 and 7) shows that the pBR325 probe hybridized to only one of the plasmid bands above the chromosomal band in the R. japonicum 191 and R. meliloti 10311 conjugants. These recombinant plasmids, which hybridized with the pBR325 probe, were quite large and therefore not well suited for use as cloning vectors. Moreover, when HB101 was transformed back with recombinant plasmids isolated from the R. japonicum 191 conjugant, it exhibited a different recombinant plasmid size (Fig. 13, panel A; lane 1). This instability of the recombinant plasmid was a further indication of its poor suitability as a vector for Rhizobium cloning experiments. Therefore, a new strategy was attempted using plasmids from a different Rhizobium strain.

Cloning of an R. meliloti 220-13 plasmids (pRm220-13) ori region into pSUP202 using the pSUP202/S17-1 system

The second strategy attempted (Fig. 14), using the mobilizable vector pSUP202 (Fig. 15) and E. coli S17-1, had substantial advantages over the pBR325/R68.45 system. Here the RP4 tra genes are inserted into the chromosome of E. coli
Fig. 14. Schematic representation of the isolation of an ori fragment from a Rhizobium meliloti 220-13 plasmid using the pSUP202/S17-1 system. A ligated mixture of pSUP202 digested with HindIII and pRm220-13 digested with HindIII was transformed into E. coli S17-1. The S17-1 transformants were then conjugated with Rhizobium. Selection was made for Rhizobium conjugants by Cm<sup>+</sup> or Am<sup>+</sup> and morphological and physiological properties.
Rhizobium host

HindIII

pSUP202

pSUP202

pSUP202
/HindIII

pSUP202-pRm220-13

RP4 tra genes

E. coli S17-1

tra genes

pSUP202-pRm220-13

S17-1 transformant

Rhizobium conjugant

ligation

transformation

conjugation

pSUP202-pRm220-13

pSUP202-pRm220-13

pSUP202-pRm220-13
Fig. 15. Genealogy of pSUP202. pBR322 was constructed by Bolivar et al. (1977) and pBR325 was derived from pBR322 by the addition of the Cm\textsuperscript{r} gene by Bolivar (1978). pSUP202 was derived from pBR325 by adding the RP4 tra gene to the plasmid by Simon et al. (1983).
pBR322 (4.3 kb)  

\[ \text{pMB1 ori} \]

Cm\textsuperscript{r} fragment

\[ \text{EcoRI} \]

pBR325 (6.0 kb)  

\[ \text{pMB1 ori} \]

RP4 Mob fragment

\[ \text{EcoRI} \]

pSUP202 (8.0 kb)  

\[ \text{pMB1 ori} \]
S17-1. This eliminates several potential problems. For example, the simultaneous transfer of both the helper plasmid and the mobilizable vector plasmid to the recipient cell cannot occur. There is also no possibility of the cointegration of the helper plasmid and mobilizable vector plasmid. *R. meliloti* 220-13 strain also has several advantages over *R. japonicum* 191 strain. *R. meliloti* 220-13 has an assortment of plasmid species which are relatively small sized. *R. meliloti* 220-13 grows very well in LB media and *R. meliloti* 220-13 plasmids were easily isolated by a standard *E. coli* plasmid isolation method (alkaline lysis method). Therefore, total *R. meliloti* 220-13 plasmid DNA was isolated and digested with various restriction endonucleases (e.g., BamHI, EcoRI, HindIII, PstI and SalI) in order to determine the resultant restriction fragment pattern in each case (Fig. 16). All enzymes, such as BamHI, EcoRI, HindIII, PstI and SalI, were utilized for this experiment according to the second strategy. However, only the experiment using HindIII gave the positive results. A mixture of *R. meliloti* 220-13 plasmids cut with HindIII and pSUP202, also cut with HindIII (within the Tet<sup>R</sup> gene), was ligated at 4°C for 12-18 hours or at room temperature (23°C) for 4-6 hours. This ligation mixture was then transformed into S17-1. The transformed cell mixture was plated onto selective media (LB agar plates containing Cm or Am). Colonies which appeared on the selective plates were patch-tested on LBCm (LB plates
Fig. 16. Restriction endonuclease analysis of *Rhizobium meliloti* 220-13 plasmids. pRm220-13 digested with *SalI* (lane 1), *BamHI* (lane 2), *HindIII* (lane 3), *EcoRI* (lane 4), *PstI* (lane 5) and pRm220-13 uncut control (lane 6). The samples were analysed on a 0.5% agarose gel with electrophoresis conditions of 30 mA (about 60 V) for 2 hours using 0.5X TBE running buffer. [Note: pRm220-13 refers collectively to all plasmids present in this strain, not to any specific individual plasmid].
containing Cm) and LBTet (LB plates containing tetracycline) simultaneously to distinguish the actual recombinants from the recircularized original vector. Colonies which survived on LBCm but not on LBTet were selected as S17-1 transformants which contained recombinant pSUP202 molecules carrying R. meliloti 220-13 plasmid inserts. Then, these S17-1 transformants were conjugated with several Rhizobium strains (R. japonicum 191, R. meliloti 10311, R. meliloti 220-13, R. meliloti 220-13-37 and R. leguminosarum 10004). Because of the dysfunction of the pSUP202 replicon inside the Rhizobium cells, recombinant pSUP202 molecules which successfully became established inside the Rhizobium conjugants should carry a replication-functional fragment of pRm220-13.

The conjugations of S17-1 carrying pSUP202-pRm220-13 recombinant plasmids with R. meliloti 10311, R. japonicum 191 and R. leguminosarum 10004 were not confirmed. However, S17-1 carrying the pSUP202-pRm220-13 library was able to successfully produce transconjugants when the original plasmid-bearing Rhizobium strain from which the cloned plasmid fragments were derived was utilized as the recipient strain. The R. meliloti 220-13 conjugants which carried pSUP202-pRm220-13 recombinant plasmids were easily obtained and separated from the donor S17-1 transformants by multiple restreaking on selective media (Cm$^R$ or Am$^R$) and by their morphological and physiological properties (Table 4). This suggested the possibility that none of the pSUP202-pRm220-13
Table 4. Comparison of morphological and physiological properties of *R. japonicum* 191, *R. meliloti* 220-13 and *E. coli* S17-1

<table>
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<tr>
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<th><em>R. japonicum</em> 191</th>
<th><em>R. meliloti</em> 220-13</th>
<th><em>E. coli</em> S17-1</th>
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<tr>
<td><strong>Physiological growth</strong></td>
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<td>1. TY medium</td>
<td>+</td>
<td>+/-</td>
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</tr>
<tr>
<td>2. LB medium</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>3. RMM medium</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>4. 30°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>5. 37°C</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
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<tr>
<td><strong>Morphological characteristics</strong></td>
<td></td>
<td></td>
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<tr>
<td>Colony morphology</td>
<td>white slimmy</td>
<td>thick yellow,</td>
<td>yellowish</td>
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<tr>
<td>on RMM</td>
<td></td>
<td>shiny, viscous</td>
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<td>with round</td>
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(+/-) indicates poor growth  
(-) indicates no growth  
(+) indicates good growth
library in S17-1 in fact carried the necessary DNA sequence(s) to be independently functional in Rhizobium and therefore required a further contribution from the original Rhizobium strain to allow successful replication. There were a number of possible explanations for such a requirement. For example, there might be a HindIII site within the minimum functional ori sequence. If so, no single HindIII fragment would confer on pSUP202 the ability to replicate in Rhizobium. Alternatively, the ori sequence may require a plasmid-encoded protein (or proteins) which is not be encoded by the HindIII fragment carrying the ori region. It should be noted that conditions such as the latter can be met by providing the necessary proteins in trans (from other plasmids or the host chromosome), while the former would require the acquisition of additional DNA sequence by the recombinant pSUP202-pRm220-13 molecule (ori sequences only function in cis). It was therefore considered a possibility that the surviving recombinant pSUP202 molecules following conjugation with R. meliloti 220-13 might be the result of a further recombination event between the donor plasmid and a resident plasmid inside the R. meliloti 220-13 (subsequent to conjugal transfer of the original recombinant pSUP202-pRm220-13 mixture to the recipient cells). Such a recombination event would obviously be facilitated by the presence of a DNA sequence in the recombinant pSUP202 (the cloned HindIII fragment) capable of homologous recombination
with the identical sequence in the resident plasmid from which it was derived. In this case the original pSUP202-pRm220-13 recombinant plasmids would act as "suicide plasmids", unable to replicate in *Rhizobium* unless a successful recombination event produced a new plasmid encoding the pSUP202 antibiotic resistance and a functional *Rhizobium* origin of replication.

It was anticipated that resident *Rhizobium* plasmids would not function as independent replicons in *E. coli*. A recombinant pSUP202 plasmid molecule would, however, be replication-functional in *E. coli*. Using this principle, total plasmids were isolated from the *R. meliloti* 220-13-37 conjugants and these were then transformed back into *E. coli* S17-1 to retrieve and verify the presence of recombinant pSUP202 plasmid molecules in the *Rhizobium* conjugants. It additionally offered a test for the presence of a shuttle vector (*E. coli*-*Rhizobium*) ability by any recombinant plasmids (Fig. 17). A recombinant pSUP202 molecule was successfully retrieved from the *R. meliloti* 220-13-37 conjugants via *E. coli* S17-1 transformation (Fig. 18). The retrieved recombinant pSUP202 molecule was cut with *HindIII* in order to determine the size of the cloned pRm220-13 *HindIII* fragment. Contrary to initial expectations, a DNA segment encompassing three *HindIII* fragments was found to be cloned into the pSUP202 vector plasmid molecule. This pSUP202-pRm220-13 recombinant plasmid molecule was named
Fig. 17. Diagramatic representation of the procedure utilized to retrieve recombinant pSUP202 plasmid molecules from Rhizobium. This is also a test of the shuttle-vector (E. coli-Rhizobium) ability of the recombinant molecules.
Step 1. Conjugation of E. coli S17-1 carrying recombinant plasmid with Rhizobium recipient

\[ \text{E. coli S17-1} \xrightarrow{\text{conjugation}} \text{Rhizobium} \]

\[ \text{pSUP202-pRm220-13} \]

Step 2. Isolation of recombinant plasmid from Rhizobium conjugant

\[ \downarrow \]

\[ \text{pSUP202-pRm220-13} \]

Step 3. Transformation of E. coli with recombinant plasmid

\[ \downarrow \]

\[ \text{E. coli transformant} \]

\[ \text{pSUP202-pRm220-13} \]

Step 4. Isolation and digestion of recombinant plasmid and agarose gel analysis of DNA fragment
Fig. 18. Comparison of pPSJ123 isolated from E. coli S17-1-123 to the plasmids isolated from R. meliloti 220-13-37 conjugant to which pPSJ123 has been introduced. pPSJ123 isolated from E. coli S17-1-123 (lane 1) and plasmids isolated from Rhizobium meliloti 220-13-37 conjugant (lane 2) are shown. E. coli S17-1-123 is the S17-1 strain which carries pPSJ123. Rhizobium meliloti 220-13-37 was created by heat-treatment at 37°C.
Restriction endonuclease mapping of pPSJ123

The restriction endonuclease cleavage map of pPSJ123 was determined for a number of enzymes including BamHI, BanII, ClaI, EcoRI, EcoRV, NcoI, PstI, SalI and XbaI. Figure 19 shows the fragment patterns of pPSJ123 when digested with various restriction endonucleases. Table 5 shows the calculated fragment sizes generated by these enzymes. This detailed restriction enzyme analysis of pPSJ123 revealed that 5 HindIII fragments were actually present (two identical 3.2 kb HindIII fragments were located at different sites in pPSJ123 and a 1.2 kb HindIII fragment which was sometimes difficult to verify due to comigration with a 1.3 kb fragment). The total size of pPSJ123 was therefore estimated to be 19.4 kb (Fig. 20).

Conjugation of S17-1 carrying individual subcloned HindIII fragments of pPSJ123 with R. meliloti 220-13

When S17-1 containing pPSJ123 was conjugated with R. meliloti 220-13, pPSJ123 was always recovered unchanged from R. meliloti 220-13 conjugants via S17-1 transformation. In order to attempt to identify the specific HindIII fragment carrying the ori region, the 1.3 kb, 2.5 kb and 3.2 kb fragments were separately subcloned into pSUP202. These subcloned plasmids were named p1 (pSUP202 carrying 1.3 kb
Fig. 19. Restriction endonuclease cleavage analysis of pPSJ123 DNA. A: pPSJ123 digested with XhoI (lane 1), PvuII (lane 2), PstI (lane 3), NcoI (lane 4), KpnI (lane 5), HpaI (lane 6), HindIII (lane 7), EcoRV (lane 8), EcoRI (lane 9), DraI (lane 10), ClaI (lane 11), BanII (lane 12), BamHI (lane 13), ApaI (lane 14) and pPSJ123 uncut control (lane 15). B: lambda digested with StyI (lane 1), pPSJ123 digested with HindIII and EcoRV (lane 2), EcoRV (lane 3), HindIII and EcoRI (lane 4), HindIII and BamHI (lane 5), pL234 digested with HindIII (lane 6), pPSJ123 digested with HindIII (lane 7), NcoI (lane 8), ClaI and EcoRI (lane 9), BamHI and NcoI (lane 10), BamHI and EcoRI (lane 11), HindIII and SalI (lane 12), SalI (lane 13), EcoRI (lane 14) and lambda digested with HindIII (lane 15).
Table 5. Resultant DNA fragment sizes when pPSJ123 is digested with various restriction endonucleases

<table>
<thead>
<tr>
<th>BamHI</th>
<th>EcoRI</th>
<th>EcoRV</th>
<th>HindIII</th>
<th>PstI</th>
<th>Sali</th>
</tr>
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<tbody>
<tr>
<td>8.2*</td>
<td>10.7</td>
<td>12.5</td>
<td>8.0</td>
<td>19.4</td>
<td>19.4</td>
</tr>
<tr>
<td>7.8</td>
<td>8.7</td>
<td>5.1</td>
<td>3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.4</td>
<td>1.8</td>
<td>2.5</td>
<td>1.3</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BamHI/EcoRI</th>
<th>BamHI/HindIII</th>
<th>EcoRI/HindIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4</td>
<td>7.6</td>
<td>4.8</td>
</tr>
<tr>
<td>4.4</td>
<td>3.0</td>
<td>3.2</td>
</tr>
<tr>
<td>3.5</td>
<td>2.5</td>
<td>2.5</td>
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<tr>
<td>3.3</td>
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<td>1.2</td>
</tr>
<tr>
<td>2.8</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EcoRV/HindIII</th>
<th>PstI/Sali</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.8</td>
<td>16.4</td>
</tr>
<tr>
<td>3.2</td>
<td>3.0</td>
</tr>
<tr>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>

* All sizes are in kb (kilobase pairs)
Fig. 20. Restriction endonuclease cleavage map of pPSJ123.
The total size of pPSJ123 is 19.4 kb. This consists of an 8.0 kb pSUP202 vector molecule, two identical 3.2 kb HindIII fragments, a 2.5 kb HindIII fragment, a 1.3 kb HindIII fragment and a 1.2 kb HindIII fragment. The 3.2 kb HindIII fragments have a single BamHI site each. The 2.5 kb HindIII fragment has only one EcoRV site. The 1.3 kb HindIII fragment has one EcoRI and one EcoRV site. The actual distance between the outermost HindIII sites of the cloned DNA segment and the vector was small but unmeasured.
pPSJ123 (19.4 kb)

- HindIII
- BamHI
- EcoRI
- EcoRV
- Cm
- Am
- Mob
- SalI
- BamHI
- EcoRV

Legend:
- 1.3 kb fragment
- 1.2 kb fragment
- pSUP202
- 3.2 kb fragment
- 2.5 kb fragment
HindIII fragment), p2 (pSUP202 carrying 2.5 kb HindIII fragment), p3 (pSUP202 carrying 3.2 kb HindIII fragment), respectively (Fig. 21) and p12 (pSUP202 carrying the 1.3 kb and 2.5 kb HindIII fragments).

In each case, when S17-1 carrying p1, S17-1 carrying p2, S17-1 carrying p3 or S17-1 carrying p12 were conjugated with R. meliloti 220-13, several additional HindIII fragments were observed in the plasmids recovered from the R. meliloti 220-13 conjugant (in addition to the original fragment). This presumably occurred as a result of recombinational events between a resident plasmid and the incoming plasmid (Fig. 22; Fig. 23). In all cases, the recovered recombinant pSUP202 molecule possessed all 3 "core" HindIII fragments found in the original pPSJ123 (1.3 kb, 2.5 kb and 3.2 kb) (Fig. 24). Therefore, these 3 fragments were considered to be the minimum required for stable independent replication.

Hybridization analysis of R. meliloti 220-13 conjugants

In order to identify which resident plasmid(s) the three "core" HindIII fragments were derived from, individual hybridization analyses of total undigested R. meliloti 220-13 plasmid DNA with these 3 fragments as probes were conducted. Figures 25, 26 and 27 show that all 3 of these fragments were derived from the 2nd largest resident plasmid (12 kb).
Fig. 21. Agarose gel analysis of DNA fragments created by digesting pPSJ123 and its subclones (p1, p2 and p3) with HindIII. HindIII digestions of p3, p2, p1 and pPSJ123 are lane 1, lane 2, lane 3 and lane 4, respectively.
Fig. 22. Agarose gel analysis of HindIII fragments from a recombinant plasmid isolated from the *E. coli* S17-1-3'. S17-1-3' was created by transforming *E. coli* S17-1 with plasmids isolated from *Rhizobium meliloti* 220-13 conjugant carrying p3. p1234 cut with HindIII (lane 1) and the new recombinant plasmid (p3') cut with HindIII (lane 2) are shown.
Fig. 23. Agarose gel analysis of HindIII fragments from a recombinant plasmid isolated from \textit{E. coli} S17-1-12'.

S17-1-12' was created by transforming \textit{E. coli} S17-1 with plasmids isolated from a \textit{Rhizobium meliloti} 220-13 conjugant carrying pl12. pl234 cut with HindIII (lane 1) and the new recombinant plasmid (pl12') cut with HindIII (lane 2) are shown.
Fig. 24. Schematic representation of agarose gel analysis of the HindIII fragments derived from recombinant plasmids. *Rhizobium meliloti* 220-13 was conjugated with each S17-1 subclone carrying recombinant pSUP202 with various HindIII fragments inserted. Following conjugation of *Rhizobium meliloti* 220-13 with the S17-1 subclones, plasmids were isolated from the *Rhizobium meliloti* 220-13 conjugants. These plasmids were then transformed back into S17-1.

Plasmids were isolated from the final S17-1 transformants. Plasmid samples were digested with HindIII and analysed by 0.7% agarose gel electrophoresis (refer to Fig. 17). The 3.2 kb HindIII fragment sometimes appears as a doublet (2 copies/plasmid). The 1.3 kb HindIII fragment also occasionally comigrates with a 1.2 kb HindIII fragment (if present).
<table>
<thead>
<tr>
<th>Recipient</th>
<th>R. <em>meliloti</em> 220-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>HindIII fragments *</td>
</tr>
<tr>
<td></td>
<td>a  b  c  d</td>
</tr>
<tr>
<td>S17-1-1</td>
<td>)  )  )  )</td>
</tr>
<tr>
<td>S17-1-2</td>
<td>)</td>
</tr>
<tr>
<td>S17-1-3</td>
<td>)</td>
</tr>
<tr>
<td></td>
<td>or</td>
</tr>
<tr>
<td>S17-1-12</td>
<td>)</td>
</tr>
<tr>
<td>S17-1-123</td>
<td>)</td>
</tr>
<tr>
<td>S17-1-1234</td>
<td>)</td>
</tr>
</tbody>
</table>

* a. 8.0 kb pSUP202;  
b. 3.2 kb HindIII fragment;  
c. 2.5 kb HindIII fragment;  
d. 1.3 kb HindIII fragment
Fig. 25. Hybridization analysis of resident Rhizobium meliloti 220-13 plasmids with the cloned 1.3 kb HindIII fragment as a radioactive probe. A (Ethidium bromide stained gel): pPSJ123 cut with HindIII (lane 1), pPSJ123 uncut (lane 2), plasmids isolated from Rhizobium meliloti 220-13 conjugant (#1) (lane 3), plasmids isolated from Rhizobium meliloti 220-13 conjugant (#2) (lane 4), plasmids isolated from Rhizobium meliloti 220-13' (lane 5). B: An autoradiogram of the same gel following Southern blotting and hybridization is shown in panel B.
A

Ethidium Bromide Stain

B

Autoradiogram
Fig. 26. Hybridization analysis of resident *Rhizobium meliloti* 220-13 plasmids with the cloned 2.5 kb *HindIII* fragment as a radioactive probe. A (Ethidium bromide stained gel): pPSJ123 cut with *HindIII* (lane 1), pPSJ123 uncut (lane 2), plasmids from *Rhizobium meliloti* 220-13 conjugant (lane 3), plasmids from *Rhizobium meliloti* 220-13-37 conjugant (lane 4) and plasmids from *Rhizobium meliloti* 220-13' (lane 5). B: An autoradiogram of the gel following Southern blotting and hybridization is shown in panel B.
Ethidium Bromide Stain

Autoradiogram
Fig. 27. Hybridization analysis of resident *Rhizobium meliloti* 220-13 plasmids with the cloned 3.2 kb *HindIII* fragment as a radioactive probe. A (Ethidium bromide stained gel): p3 cut with *HindIII* (lane 1), pPSJ123 uncut (lane 2), plasmids from *Rhizobium meliloti* 220-13 conjugant #2 (lane 3), plasmids from *Rhizobium meliloti* 220-13-37 conjugant (lane 4) and plasmid from *Rhizobium meliloti* 220-13' (lane 5). B: An autoradiogram of the gel following Southern blotting and hybridization is shown in panel B.
A
Ethidium Bromide Stain

B
Autoradiogram
Curing of *R. meliloti* 220-13 plasmids

There are several methods involving chemical treatment (e.g., with SDS, EtBr, acridine orange or mitomycin C) which have been shown to cure bacterial cells of plasmids (Hynes et al. 1989; Skogen-Hagenson and Atherly 1983). All the methods mentioned above were applied to *Rhizobium* strains. However, culturing plasmid-bearing strains at elevated temperatures (37°C incubation for *Rhizobium*) was the only treatment to have an effect on the plasmid content of *R. meliloti* 220-13. As seen in Figure 28, several different plasmid band patterns were obtained from *R. meliloti* 220-13 isolates following treatment at 37°C. Although most of the resident plasmids could be eliminated by heat treatment, a complete loss of plasmids from *R. meliloti* 220-13 was not observed even following 24 repetitions of subculture at 37°C. Extensive heat treatment reduced the number of observed plasmids to one (Fig. 28, panel C; lane 1), [this strain was designated as *Rhizobium meliloti* 220-13-37 and used as the recipient *Rhizobium* strain for specified conjugation experiments], but several plasmid bands reappeared following subsequent culture at 30°C. This suggested that high temperature treatment suppressed the plasmid replication to a minimum level while the strain was at 37°C, but that the plasmids could be subsequently recovered during growth at 30°C.

Transformation and electroporation of *R. meliloti*
Fig. 28. Plasmid band patterns from *Rhizobium meliloti* 220-13 strains after heat treatment (37°C culture). Plasmid patterns for 3 independent treatments are shown (panels A-C). A: plasmid band patterns from *Rhizobium meliloti* 220-13 strains following heat treatment (lanes 1 and 2), plasmids from *Rhizobium meliloti* 220-13 control (lane 3). B: plasmid band patterns from *Rhizobium meliloti* 220-13 strains following heat treatment (lanes 1, 2, 3 and 4), plasmids from *Rhizobium meliloti* 220-13 control (lane 5). C: plasmids from *Rhizobium meliloti* 220-13 strains following heat treatment (lane 1) [this strain was thereafter designated as *Rhizobium meliloti* 220-13-37], plasmids from *Rhizobium meliloti* 220-13 control (lane 2).
A

pRM220-13 from heat-treated strain

1 2 3

B

pRM220-13

1 2 3 4 5

C

pRM220-13

1 2
**220-13 with pPSJ123**

Several *Rhizobium* transformation methods, such as freezing-thawing (Selvaraj and Iyer 1981), competence medium (Raina and Modi 1969) and CaCl$_2$-MgCl$_2$ treatment (Kiss and Kalman 1982) were utilized in attempts to transform *R. meliloti* 220-13. After numerous attempts, none of these methods gave acceptable levels of *R. meliloti* 220-13 transformation using a variety of plasmids. However, *R. meliloti* 220-13 was successfully transformed with pPSJ123 isolated from *R. meliloti* 220-13 conjugants by electroporation (Fig. 29), but not pPSJ123 isolated from *E. coli* S17-1 (Table 6). Other *Rhizobium* strains were recalcitrant to transformation with even pPSJ123 isolated from *R. meliloti* 220-13. This observations suggest that the transformation difficulties with *Rhizobium* may be at least partially explained by the presence of restriction and modification barriers.

**Cloning of the parathion hydrolase gene (PH) into pPSJ123 and pl**

The multiple step protocol diagrammed in Figure 30 was successfully utilized to clone the parathion hydrolase gene (1.5 kb) into pPSJ123. The DNA fragment encoding the parathion hydrolase gene was isolated from pCMS40 with BamHI "sticky ends". In order to insert this into the unique *PstI* site (in the Am$^R$ gene) of pPSJ123, the BamHI ends of the
Fig. 29. Plasmid band patterns of *Rhizobium meliloti* 220-13 electro-transformants carrying pPSJ123. pPSJ123 (lane 1), plasmids from *Rhizobium meliloti* 220-13 electro-transformants (lane 2 and lane 3) and plasmids from *R. meliloti* 220-13' (lane 4).
1: pPSJ123

2: pPSJ123

3: isolate from electro-transformant

4: pRM220-13'
Table 6. Electroporation of *Rhizobium* strains with pPSJ123

<table>
<thead>
<tr>
<th><em>Rhizobium meliloti</em> 220-13</th>
<th><em>Rhizobium meliloti</em> 10311</th>
<th><em>Rhizobium japonicum</em> 191</th>
<th><em>Rhizobium leguminosarum</em> 10004</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPSJ123 from <em>Rhizobium</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pPSJ123 from <em>S17-1</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(+) indicates successfully electro-transformed

(-) indicates no electro-transformants obtained
Fig. 30. Schematic representation of the procedure utilized to modify the BamHI ends of the parathion hydrolase gene fragment to PstI cohesive ends using BamHI-PstI adaptors.
Step 1. BamHI-PstI adaptor phosphorylation

5'OH GATCCCTGCAGG 3'OH
3'OH GGACGTCCCTAG 5'OH

↓

Step 2. Ligation of BamHI-PstI adaptor to Parathion Hydrolase (PH) gene fragment

5'P BamHI

adaptor

PH

adaptor

PH 1.5 kb

5'P BamHI

↓

Step 3. Gel electrophoresis to eliminate the unused adaptors

↓

Step 4. Electroelution of adaptored PH

5'P BamHI

PH

PstI

5'P BamHI

↓
Step 5. PstI digestion of adapted PH and ligation with pPSJ123 cut with PstI

Step 6. Transformation of S17-1 with ligation mixture of PH and pPSJ123
parathion hydrolase gene fragment were modified to convert them to PstI cohesive ends using BamHI-PstI adaptors. After adding the BamHI-PstI adaptors to the parathion hydrolase gene fragment, unused adaptor molecules were eliminated from the ligation mixture by agarose gel electrophoresis. Then, the BamHI-PstI adaptor-modified parathion hydrolase gene fragment was eluted from the gel slice and cut with PstI to provide the desired PstI cohesive ends. This fragment was mixed with pPSJ123 previously cut with PstI. The two molecules were ligated with T4 ligase at room temperature (23°C) for 6 hours. The ligation mixture was then transformed into E. coli S17-1. Transformants were selected on LBCm plates. The plasmid content of transformants was checked from rapid isolates cut with PstI to release any cloned fragments. Figure 31, lane 2 shows that the PstI DNA fragment encoding the parathion hydrolase gene which was cloned into pPSJ123 was somewhat smaller than the original BamHI parathion hydrolase gene fragment. This is as expected since the original BamHI fragment possesses a PstI site about 200 bp in from the 5' end (5' with respect to the direction of parathion hydrolase gene expression). However, this does not affect parathion hydrolase gene activity because the actual translational start signal is located about 300 bp in from the 5' end of this BamHI fragment (Serdar 1989).

The parathion hydrolase gene was cloned into pl in order to evaluate the relative efficiency of a different promoter
Fig. 31. Agarose gel electrophoretic analysis of recombinant pPSJ123 and pl molecules carrying a gene fragment encoding parathion hydrolase. pPSJ123-PH from S17-1 (lane 1), pPSJ123-PH digested with PstI (lane 2), p1-PH digested with BamHI (lane 3) and pCMS40 digested with BamHI (lane 4). The electrophoresis conditions were 100 V (30 mA) for 1 hour, a 0.7% agarose gel and 0.5X TBE running buffer.
activity of the cloning vector (the promoter of the Tet gene) with regard to expression parathion hydrolase activity. The gene fragment encoding parathion hydrolase was isolated from pCMS40 by digesting the plasmid with BamHI. This was ligated with pl (also cut with BamHI within the Tet gene). The ligation mixture was then transformed into E. coli S17-1 and transformants were selected on LBCm or LBAm. Figure 31, lane 3 shows the cloned parathion hydrolase gene fragment in pl.

**Parathion hydrolase gene activity in R. meliloti 220-13 conjugants**

*R. meliloti* 220-13 conjugants carrying the parathion hydrolase gene were created by conjugation of the Rhizobium with *E. coli* S17-1 carrying pPSJ123-PH or pl-PH. However, when parathion hydrolase gene activity was assayed by the colorimetric method of Serdar (1985), no yellow color development (as the measurement of gene expression) was observed in either of the *R. meliloti* 220-13 conjugants.

When the parathion hydrolase gene was under the control of the lac promoter in pUC7, it was expressed very well in *E. coli* HB101 or *E. coli* S17-1 hosts. However, when it was under the control of the Am promoter in pPSJ123, it was not expressed at a detectable level even in *E. coli* S17-1. The parathion hydrolase gene subcloned into pl under the Tet promoter was also not expressed in Rhizobium or *E. coli* S17-1.
host.

Several factors, such as plasmid copy number, differential promoter activity and recognition, ribosome binding sites and appropriate growth conditions could affect gene expression independently or collectively with any of the others. Even the elimination of the antibiotic resistance marker, which should have very little to do with the level of expression of an unrelated gene product, could have lead to plasmid instability and ultimately affected expression.

Summary

The goal of this project was to construct a cloning vehicle for the stable transformation of rhizobia based upon a native *Rhizobium* plasmid origin of replication. The conjugable *E. coli*-Rhizobium shuttle vector pPSJ123 was constructed from the *E. coli* plasmid pSUP202 and a portion of the second largest resident plasmid of *Rhizobium meliloti* 220-13. This vector construct can be readily transferred to the *R. meliloti* 220-13 strain by conjugation and back to *E. coli* by transformation. The inability of the vector to be transferred to other Rhizobia could reflect the presence of restriction barriers for these strains. However, it might also be indicative of a further required contribution from the *R. meliloti* 220-13 (or its plasmids) to the replication
of this vector. A continued involvement of resident plasmids could not be ruled out due to the difficulty in producing a stable cured *R. meliloti* 220-13 strain. The relatively large segment of *Rhizobium* DNA incorporated into all of the pPSJ123 family of plasmids suggests that the region encodes more than a simple ori sequence (generally less than 500 bp). At this time the minimum length of *Rhizobium* DNA present in these vectors is about 7 kb. Although no data confirming open reading frames in this region is available, it is suspected that a replication protein is encoded by a portion of this fragment. The total minimum vector size is about 15 kb at this time, a reasonable size for such a cloning vehicle.

Future refinements of the pPSJ123 family of cloning vehicles are possible. The insertion of a multiple cloning site would increase the number of restriction fragment types which could be readily inserted. This would be particularly useful if it included a β-galactosidase complementation system such as that found in the pUC series plasmids or similar color based selective marker. A determination of the nucleotide sequence of the 7 kb *Rhizobium* DNA fragment would undoubtedly make a reduction in the size of the cloning vector possible. Open reading frames and potential regulatory sequences could then be identified and individually evaluated as to their importance with regard to stable replication. Although further refinements of the pPSJ123 plasmids will be possible in the future, the present plasmid was successfully utilized
to introduce a foreign gene into *R. meliloti* 220-13. Even though the gene was not expressed, it was maintained by the *Rhizobium* cells.
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