STUDIES ON THE PLASMID-CODING OF NODULATION AND NITROGEN FIXATION GENES IN TWO STRAINS OF *RHIZOBIUM TRIFOLII*

By

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ABSTRACT

STUDIES ON THE PLASMID-CODING OF NODULATION AND NITROGEN FIXATION GENES IN TWO STRAINS OF *RHIZOBIUM TRIFOLII*

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Large plasmids coding for genes essential for the *Rhizobium trifolii*-clover symbiosis have been identified. Transfer of pJB5JI, a *R. leguminosarum* plasmid coding for pea nodulation (*nod*) and the nitrogenase (*nif*) structural genes, into *R. trifolii* strain T37 generated transconjugants containing a variety of plasmid profiles formed from the recombination of pJB5JI and pRtT37a. The symbiotic properties exhibited on both hosts and the plasmid profiles were stably maintained even after reisolation from root nodules. Approximately 30% of the transconjugants, which contained a "hybrid" plasmid corresponding in size to pJB5JI, formed effective (nitrogen-fixing) nodules on peas but were unable to nodulate clover. This suggests that the *R. trifolii nod* genes had been deleted. Hybridization analysis indicated that the *R. trifolii nif* genes, which are located on pRtT37a, had also been deleted. Another 40% of the transconjugants harbored "hybrid" plasmids of various molecular weights. These strains contained the *R. trifolii nif* genes and formed effective nodules on clover. However, these strains lacked the *R. leguminosarum nif* genes and formed ineffective

nodules on peas. This suggests that the R. trifolii nif genes were unable to complement the deleted R. leguminosarum nif genes in pea nodules. The remaining 30% of the transconjugants contained both sets of symbiotic genes and formed effective nodules on both peas and clover. Results from the analysis of the strain T37 (pJB5JI) transconjugants indicated that genes essential for clover nodulation and nitrogen fixation are encoded on pRtT37a. The linkage of nif and nod genes on a plasmid in R. trifolii strain 0403 was demonstrated. Analysis of plasmid DNA from a spontaneous non-nodulating mutant of strain 0403 indicated that about 70 Mdal of DNA had been deleted from pRt0403a, the smallest plasmid in this strain. This non-nodulating mutant was able to attach to clover root hairs, but could not induce the formation of infection threads, suggesting that genes essential for infection thread formation had been lost. Southern hybridization analysis indicated that the R. trifolii nif genes which are encoded on pRt0403a, had been deleted. Thus, the nif genes and genes essential for nodulation are linked on a 70 Mdal region of pRt0403a DNA.

To Mom and Dad

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

amp^r	ampicillin resistance
BSA	bovine serum albumin
CCC	covalently closed circular
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	(ethylenedinitrilo)-tetraacetic acid, disodium
EtBr	ethidium bromide
EtOH	ethyl alcohol
FeMo-co	iron-molybdenum cofactor
Fix	nitrogen fixation phenotype
gln	glutamine regulatory gene
IS	insertion sequence
kan^r	kanamycin resistance
kb	kilobase
Mdal	megadalton
µCi	micro Curie
µE	micro Einstein
nif	nitrogen fixation genes
Nod	nodulation phenotype
nod	nodulation gene
ntr	nitrogen regulatory gene

OC	open circular, relaxed
ori	origin of replication
PEG	polyethylene glycol
pfu	plaque-forming units
phe	phenylalanine auxotroph
POPOP	1,4-bis[2-(4-methyl-5-phenyloxazoyl)]-benzene
PPO	2,5-diphenyloxazole
PVP	polyvinylpyrrolidone
rif^r	rifampicin resistance
RNase	ribonuclease
rpm	revolutions per minute
SiDi	standard deviation
SDS	sodium dodecyl sulfate
str	streptomycin resistance
sym	symbiotic gene
tet^r	tetracycline resistance
TCA	trichloroacetic acid
Tn	transposon
Tris	Tris(hydroxymethyl)aminoethane
trp	tryptophan auxotroph
UV	ultraviolet
V	volume
W	weight
w.t.	wild-type

INTRODUCTION

Scientists throughout the world are confronted with the task of increasing agricultural productivity to feed an expanding world population. Past increases in food production can be attributed primarily to the greater use of mechanization, improved crop species, and chemicals, such as herbicides, pesticides, and nitrogenous fertilizer. In fact, the majority of the increases in productivity of cereal crops over the last twenty years have resulted from increased applications of nitrogenous fertilizer (77,182).

The production of nitrogenous fertilizer, however, is an energy intensive process. The synthesis of ammonia from dinitrogen is carried out under high pressure and temperature by the Haber-Bosch process. This industrial process requires a vast input of energy, usually from natural gas. Currently 6% of the seven trillion cubic feet of natural gas consumed by industrial processes in the United States is used for the production of nitrogenous fertilizer (1). The natural gas used for feedstock and fuel for ammonia synthesis is estimated to account for 83% of the production cost by 1985 (1). For this reason, the cost of nitrogenous fertilizer is closely correlated with the cost and availability of natural gas. The supply of natural gas is not inexhaustible, however, and is subject to possible interruptions due to the current partial dependence upon foreign sources. These disadvantages of industrially produced nitrogenous fertilizer (i.e., high cost,

unstable energy supply) have aroused great interest in the search for alternative technologies.

Some prokaryotic organisms can convert dinitrogen to ammonia for growth. This process, termed biological nitrogen fixation, occurs in a diverse group of prokaryotes. Some bacteria, such as Azotobacter, fix dinitrogen under aerobic conditions, while others (e.g., Klebsiella and Clostridium) fix dinitrogen anaerobically. Another genus of bacteria, Rhizobium, normally fixes dinitrogen only in a species-specific symbiotic association with leguminous plants, such as clover, soybeans, and peas. The symbiosis is beneficial to the plant in that all of the nitrogen required for plant growth can be supplied by the Rhizobium. In return, the host plant provides the bacteria with all of their nutrient requirements. Thus, the energy-intensive production and application of nitrogenous fertilizer for plant growth is obviated.

The extension of biological nitrogen fixation to important food crops, such as the cereals, corn, wheat, and rice, is therefore an obvious, albeit, long-range goal. A prerequisite for the extension of symbiotic nitrogen fixation to other crops would be a better understanding of the genetic and biochemical events involved in the establishment and maintenance of the Rhizobium - legume symbiosis.

The establishment of the symbiosis involves the specific recognition between the species of Rhizobium and the host legume root (6,43, 44). This recognition is proposed to be mediated by the interaction between host plant lectins and specific residues in the Rhizobium cell wall polysaccharide (16,20,48,74,207). After attachment to the root hair, the Rhizobium invade the root hair via a structure called the infection thread (43,44).

Tumor-like growths, termed nodules, develop on the legume root at some of the infection sites (43,44). Within the nodule, bacteria are released from the infection threads into membrane vesicles in the nodule cell cytoplasm. The bacteria then differentiate into bacteroids, the form in which biological nitrogen fixation occurs.

At the time this research was initiated, several reports indicated that genes essential for nodulation (*nod*) and nitrogen fixation (*nif*) might be located on plasmid DNA in strains of *Rhizobium*. Culturing *Rhizobium* under certain growth conditions or in certain soils resulted in a loss of nodulation ability (59,175,187). Treatment of strains of *Rhizobium* with plasmid "curing" agents, such as intercalating dyes or SDS, also resulted in the loss of nodulation ability (59,85,216). Dunican and Tierney (60) observed conjugal transfer of *nif* genes from *R. trifolii* to a *nif*$^-$ strain of *K. aerogenes*, and suggested that this was plasmid mediated. Although all of these studies suggested that plasmids might be involved in the formation of the symbiosis, there was no direct evidence for the presence of plasmid DNA in *Rhizobium*.

To address this problem, experiments were designed to determine whether plasmids were present in *Rhizobium* and, if so, whether the *nif* genes and/or *nod* genes were plasmid-encoded.

In Chapter I, preliminary experiments are described for the detection, isolation, and characterization of plasmid DNA in strains of *Rhizobium*. Plasmid "curing" experiments were carried out, and isolates from the treated cultures were examined for the loss of symbiotic properties and of a specific plasmid.

In Chapter II, the identification of a plasmid (pRtT37a) in *R. trifolii* strain T37 which encodes the *nif* structural genes and genes

essential for clover nodulation is reported. This was accomplished by the characterization of plasmids resulting from the interaction between pJB5JI, a R. leguminosarum plasmid, and pRtT37a, and by direct hybridization of a heterologous nif probe to a Southern filter of pRtT37a DNA.

In Chapter III, the characterization of a non-nodulating mutant of R. trifolii strain 0403 which resulted from the spontaneous deletion of plasmid DNA is described. The nif and nod genes were localized to a specific plasmid (pRt0403a) in R. trifolii strain 0403.

LITERATURE REVIEW

Biological Nitrogen Fixation. The process whereby dinitrogen is enzymatically converted to ammonia is termed biological nitrogen fixation. This process occurs only in prokaryotic organisms, both free-living and symbiotic nitrogen-fixing bacteria. The genetics and biochemistry of nitrogen fixation have been extensively studied in the free-living organisms (25,26,131). However, the genetic and biochemical events involved in the establishment and maintenance of an effective (nitrogen-fixing) symbiosis between Rhizobium and leguminous plants are only now being elucidated.

Rhizobia are aerobic, gram-negative, rod-shaped (0.5 to 0.9 x 1.2 to 3.0 μm) soil bacteria which proliferate in the rhizosphere of potential host plants (2,43,44,173). Rhizobia are capable of forming a species-specific symbiosis with legumes, some of which are agronomically important. The specificity of the symbiotic association is the basis for the classification of Rhizobium. Seven species of Rhizobium and their host plants are listed in Table 1.

The establishment of the Rhizobium-legume symbiosis involves a complex series of steps (see 6,43 and 44 for reviews). Initially, the host plant must recognize the correct species of Rhizobium among the many soil bacteria. This recognition and attachment step has been suggested to be mediated by plant lectins, proteins which bind specific carbohydrate moieties (16,20,48,74,114,207). Rhizobium induces curling

Table 1. Classification of *Rhizobium* and their hosts[a]

Host group	Species	Growth rate[b]	Agronomically important host plant
Pea and vetch	*R. leguminosarum*	Fast	*Pisum arvense* (field pea) *Pisum sativum* (garden pea) *Vicia faba* (broad bean) *Vicia sativa* (common vetch) *Lens culinaris* (lentil) *Lathyrus odoratus* (sweet pea)
Bean	*R. phaseoli*	Fast	*Phaseolus vulgaris* (garden bean)
Clover	*R. trifolii*	Fast	*Trifolium* spp. (clover)
Alfalfa	*R. meliloti*	Fast	*Medicago sativa* (alfalfa)
Soybean	*R. japonicum*	Slow	*Glycine max* (soybean)
Cowpea	*R.* "cowpea"	Slow	*Vigna unguiculata* (cowpea) *Phaseolus lunatus* (lima bean) *Vigna radiata* (mung bean) *Cajanus cajan* (pigeon pea) *Arachis hypogaea* (peanut)
Lupin	*R. lupini*	Slow	*Lupinus* spp. (lupinus) *Ornithopus sativus* (serradella)

[a]Data were condensed from Schubert (173).
[b]Fast growth rate indicates a generation time of 4 to 6 h; slow growth rate indicates a generation time of 8 to 10 h.

and deformation of root hairs of its host plant, and also the development of a tubular structure, termed the infection thread (6,27,43,44, 212). Rhizobia are carried with the infection thread as it elongates toward the root cortex. Plant cells in the root cortex are stimulated to divide and enlarge, thus producing the nodule. When the infection thread reaches the root cortex, the bacteria are released from the infection thread into membrane vesicles (44). The bacteria then differentiate into bacteroids, the symbiotic form which carries out biological nitrogen fixation (114). These steps will be discussed in more depth in the next sections.

Lectin Recognition Hypothesis. One aspect of the Rhizobium - legume symbiosis is the specificity whereby a particular legume is nodulated only by a certain species of Rhizobium. The legume must, in some fashion, recognize the correct microsymbiont (Rhizobium species) in the soil microflora, and permit its entry to the exclusion of other, possibly harmful, microorganisms.

Recently, plant lectins (phytolectins, phytohemaglutinins) have been proposed to play an important role in the recognition and binding between Rhizobium and the host root (16,20,48,74,207). Lectins are a group of proteins, found in both plants and animals, which have the capability of binding carbohydrates and carbohydrate-containing molecules in a highly specific fashion (for review, see 115). In most species of plants, the highest concentrations of lectins are found in the seed, although lectins may also be present to a lesser extent in the roots, stems and leaves (194).

The physiological role lectins play in the plant is not entirely clear at present. One propbsed role for plant lectins in legumes is

the mediation of the selective interaction between legume roots and the Rhizobium cell wall during the initial stages of the infection (20,48, 74). Hamblin and Kent (74) reported that phytohemagglutinin (PHA) from seeds of Phaseolus could agglutinate R. phaseoli cells. Production of PHA by mature roots of Phaseolus was also observed. The binding of R. phaseoli cells to sites on the roots suitable for infection was suggested to be mediated by PHA.

A specific interaction between FITC-conjugated soybean lectin and all but three of twenty-five strains of R. japonicum has also been reported (20). The lectin did not bind to 23 heterologous strains of Rhizobium which do not nodulate soybeans. Subsequent studies in another laboratory have achieved similar results (16).

Wolpert and Albersheim (207) and Kamberger (103) have reported a specific interaction between legume lectins and isolated cell surface polysaccharides from several Rhizobium strains. Lectins isolated from seeds of a legume apparently bind to the polysaccharide from the specific strain of Rhizobium capable of nodulating the host legume. No interaction was observed between Rhizobium polysaccharides and lectins from non-host plants.

Dazzo and Hubbell (48) have proposed a simple model for the basis of host specificity. The clover lectin, trifoliin, is proposed to form a cross-bridge between R. trifolii and clover root hairs. The binding of the lectin occurs to cross-reactive antigenic sites on the bacteria and the clover root hair (48). These antigenic sites appear to be localized to the tips of clover root hairs (46), and have a transient appearance on the cell surface of R. trifolii (49,96).

Some results obtained cast doubt upon the role of lectins in the infection process. Some strains of R. japonicum formed effective nodules on soybeans, but were unable to bind soybean lectin (128). This may reflect a transient appearance of the lectin receptor on the cell surface of R. japonicum as has been reported for R. trifolii (49, 96).

Wong (208) has observed that binding of the legume lectin to a Rhizobium strain does not necessarily mean that the strain will nodulate the legume. Concanavalin A, the jack bean lectin, bound to all Rhizobium strains tested, although only one strain could nodulate jack beans (208). Lack of binding of host legume lectin to some Rhizobium strains which nodulate lentils, peas, and broad beans was also observed (208). This may again be due to the transient nature of the lectin receptor (16,49,96).

A number of soybean lines which lack lectin in the seeds and in the roots have been found (151,188). These soybean lines can still be effectively nodulated. Whether there is a different non-cross reactive lectin in the roots of these soybeans which would mediate binding of R. japonicum is not known.

The inconsistent results and gaps in the data make defining a clear role for lectins in the recognition step of the Rhizobium - legume symbiosis difficult. Hopefully, further research on this problem will result in a better understanding of how the recognition between the host legume and Rhizobium is mediated.

Role of Bacterial Polysaccharides. Cell surface polysaccharides of Rhizobium are probably important in the establishment of the symbiosis, in conjunction with the proposed role for legume lectins (15,16, 20,48,49,120,133,180,207). A number of different cell surface

polysaccharides have been described for Rhizobium: exopolysaccharides (EPS), capsular polysaccharides (CPS), lipopolysaccharides (LPS), and various glucans, including cellulose (6).

Structural analyses of exopolysaccharide (EPS) isolated from R. trifolii have shown a repeating unit of eight glycosyl residues (6,99, 162). However, 2-deoxyglucose, the specific sugar hapten for the clover lectin, was not detected. This suggests that either the EPS was not the polysaccharide which mediates the specific recognition between R. trifolii and clover root hairs, or that the model (48) proposing a cross-bridging role for clover lectin between the plant root hair and a bacterial polysaccharide is incorrect.

The EPS on which structural analysis was performed was isolated from cultures in the phase of growth which Dazzo et al reported (49) lacked the ability to bind clover lectin. However, possible tests for biological activity of the purified EPS such as lectin binding, induction of root hair curling, etc., were not carried out.

Several polysaccharides from R. trifolii have been reported to possess biological activity. Dazzo et al. (49) suggested that the transient clover lectin receptor in R. trifolii was a capsular polysaccharide. Another report by the same workers found that the biologically active polysaccharide of R. trifolii exhibits the characteristics of lipopolysaccharides (47). Thus, the exact nature of the biologically functional polysaccharide of R. trifolii is still questionable.

The exopolysaccharide (EPS) of R. japonicum has been reported to react in a biologically specific manner with soybean lectin (16,196). Kamberger (103) observed an interaction between soybean lectin and R. japonicum EPS but not between the lectin and the lipopolysaccharide of

R. japonicum. Others, however, have observed interactions between Rhizobium LPS and the host plant lectin (6,47,103,145,207). The purity of the LPS used in these studies was questionable, as was the specificity of binding since sugar hapten controls were not employed. Highly purified LPS has been obtained from several strains of Rhizobium but has not been tested for biological activity (6). The sugar composition of the LPS from these Rhizobium strains varied as much between strains as between species. How the great variability in the composition of LPS would mediate the specificity of the legume-Rhizobium symbiosis is not clear at present.

Plant Cell Responses. I. Root Hair Curling. The first observable plant response during the establishment of the symbiosis is root hair curling or deformation (6,27,43,44). This response can be induced with heterologous strains of Rhizobium, but the response is not as great as observed with homologous strains (44,109).

The compound(s) responsible for the induction of root hair curling has not yet been elucidated. Sterile filtrates of liquid cultures of Rhizobium induce the typical root hair deformations, but not to the same extent as live cells (97,184,211,212). Indole acetic acid (IAA) has been implicated in the curling phenomenon since IAA can be synthesized by Rhizobium (43,44). However, root hair deformations caused by IAA are distinctly different than those observed with live Rhizobium cells (6,43,44).

Hubbell (97) has reported that the curling inducer(s) for clover root hair curling is present in a crude extracellular polysaccharide preparation. The curling agent(s) which was dialysable and heat labile was not characterized further. Solheim and Raa (184) observed two

fractions of culture filtrate which caused root hair curling. One fraction was sensitive to nucleases and the other was believed to be a polysaccharide or protein. Similar results have also been reported by Yao and Vincent (212).

The function of root hair curling in the infection process has not been well established. While infections generally occur on curled root hairs, there is no evidence that curling is a prerequisite for infection. Indeed, several reports have indicated that infection of legume root hairs by Rhizobium cells can occur on straight root hairs (45,143, 163). The curling of the root hair tip may result in the entrapment of the Rhizobium cells. This might present a greater opportunity for infection to occur (134,163).

Bauer recently proposed a speculative mechanism for root hair curling (6). The root hair cell wall appears to be composed of two layers (43,44). The outer layer is flexible while the inner layer which does not extend over the growing tip region is rigid and fibrillar (43). Attachment of Rhizobium cells is proposed to result in localized inhibition of synthesis of the inner rigid layer of the root hair cell wall. As the root hair continues to elongate, the tip curls around the Rhizobium attachment site. This eventually results in envelopment of the attached Rhizobium cells between the root hair cell walls.

Plant Cell Responses: II. Infection Thread Formation.

The bacteria invade the root hairs and are enclosed in a structure known as the infection thread. This is a tubular structure which elongates towards the base of the root hair and through preexisting plant cell walls as it advances into the root cortex (6,43,44). The

infection thread may branch several times before reaching the root cortex (43,44).

The infection thread appears to result from an invagination of the root hair cell plasma membrane and cell wall (133,163). The wall of the infection thread has been shown in electron microscopic studies to be contiguous with the root hair cell wall (84,134,169). Histochemical studies have shown that the infection thread is composed of the same components as the root hair cell wall (44).

The event(s) which triggers the initiation of the infection thread is unknown, although mechanical rupture or enzymatic degradation of the root hair cell wall, or the entrapment of Rhizobium cells by the curled root hair have been suggested (43,44). In the rare cases where infections occur in non-curled root hairs, the initiation of infection threads may occur by attached Rhizobium cells at the point of contact of two non-curled root hairs (6).

The Rhizobium cells enter the infection thread and swim freely and multiply within it. As the infection thread branches and elongates towards the root cortex, Rhizobium cells are carried (or swim) along. When the infection thread reaches the root cortex, the Rhizobium cells are released into the cytoplasm of root cortex cells in a membrane vesicle derived from the infection thread, termed the peribacteroid envelope (44).

Bacteroid Formation and Nodule Development. As the branching infection thread elongates into the root, proliferation of root cortical tissue cells occurs (44,114). The branches of the infection thread penetrate into this meristematic region and the bacteria are released into the cortical cells (43,44). The bacteria multiply within the

peribacteroid envelope and begin to differentiate into the symbiotic form called bacteroids. This differentiation process results in little morphological changes in R. japonicum and R. "cowpea" bacteroid cells, whereas, R. leguminosarum and R. trifolii cells form branched and lobed structures up to forty times larger than the free-living bacterial cells (114).

New gene products are also expressed in the bacteroids during and following the differentiation process. The nif gene products, dinitrogenase and dinitrogenase reductase, are expressed in the bacteroid state. Dinitrogenase, a soluble iron-molybdenum protein, is a tetramer ($\alpha_2\beta_2$) of two different subunit types (62,131). The enzyme contains a small iron-molybdenum cofactor (FeMo-co) which has been suggested to be the active site for the reduction of dinitrogen to ammonia (153,180). Dinitrogenase reductase is a non-heme iron protein composed of two identical subunits (62,131). Dinitrogenase reductase transfers electrons for reduction of substrate from a flavodoxin and/or ferridoxin to dinitrogenase during nitrogen fixation (131).

Changes in the cytochrome composition also occur during differentiation of bacteria to bacteroids. Cytochromes a, a_3, and o are expressed in bacteria but not in bacteroids (8). These cytochromes are replaced in the bacteroids by several new cytochromes, including cytochrome c (552), P-450, and P-420. This change in the composition of electron transport proteins may reflect the adaptation of the bacteroid to the low oxygen concentration which exists in the root nodule (8).

The activity of the heme biosynthetic enzymes increases dramatically during nodule development (132). The heme produced is the

prosthetic group of leghemoglobin, an oxygen binding protein present at high concentrations in legume root nodules.

The genetic and biochemical role of the host legume in the symbiosis is only now being elucidated. Classical genetic studies have indicated that a number of plant genes are involved in nodule development and the formation of an effective (nitrogen-fixing) symbiosis (32,92, 140). Apart from leghemoglobin, the identity and function of these gene products is unknown.

Leghemoglobin is truly a unique protein. The legume host synthesizes the globin apoprotein (28,53) while Rhizobium bacteroids synthesize the heme moiety (42,132). Leghemoglobin appears to be located in the plant cell cytoplasm (199,200) and not inside the peribacteroid envelope with the Rhizobium bacteroids, as previously reported (9,160).

The function of the leghemoglobin appears to be the regulation of oxygen tension in the root nodule (3,206). The high concentration of leghemoglobin in nodules (1 mM in soybean nodules) reduces the concentration of free oxygen to less than 10 nM (3). The dinitrogenase enzyme complex, which is sensitive to oxygen inactivation, thus remains active. A high flux of oxygen to the respiring bacteroids is also maintained by the high concentration of leghemoglobin.

Legocki and Verma (112,113) have detected at least 18 to 20 polypeptides in addition to leghemoglobin which are specific to soybean root nodules. These nodule-specific proteins, termed nodulins, are synthesized by the host plant. Nodulins account for about 10% of the [^{35}S] methionine-labeled protein synthesized in the host cell cytoplasm. The proteins are not detected in uninfected roots, and appear to be plant gene products necessary for the development and maintenance

of the root nodule. Most of the nodulins have molecular weights between 12,000 and 20,000 (113), while one nodulin of unknown function has a molecular weight of 35,000 (112).

Nodules which develop from inoculation of soybeans with ineffective (Fix$^-$) strains of R. japonicum generally contain lower levels of the nodulins (112). Differential expression of nodulins was observed in nodules of soybeans inoculated with different mutant strains of R. japonicum. At present, the mechanism by which R. japonicum (and mutant strains) influence the expression of the host nodulin genes is not known.

Organization and Regulation of the nif Genes. Genetic analysis of the nif genes in strains of Rhizobium has been difficult since these strains normally fix nitrogen only during the symbiosis with the legume plant. Most of the genetic analyses of the nif genes have been performed on the free-living, nitrogen-fixing bacterium, K. pneumoniae.

The nif operon in K. pneumoniae consists of at least 17 contiguous genes located on a 24 kb segment of the chromosome (26,160). The nif genes are arranged into 7 or 8 operons which are transcribed in the same direction (Figure 1). Many of the nif gene products have been identified and functions have been assigned for some. These data are summarized in Table 2.

The nif H gene codes for the iron-containing protein, dinitrogenase reductase (161). The gene product of nif H is processed by the products of nif M and nif S to yield the active protein (150,159,161). The α- and β-subunits of dinitrogenase, the FeMo proteins, are coded for by nif D and nif K, respectively (161). An iron-molybdenum cofactor (FeMo-co) is synthesized and processed by the gene products of nif

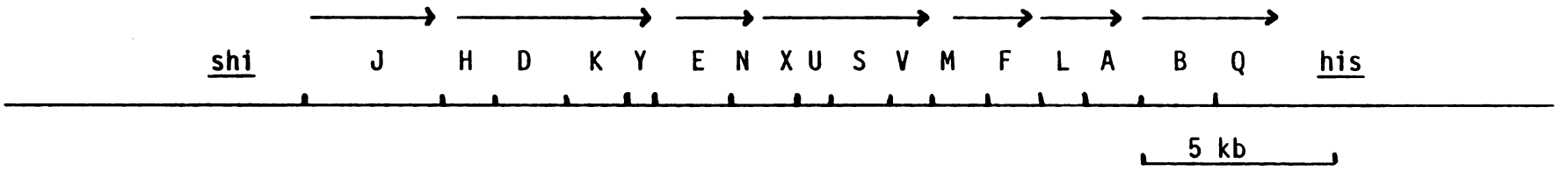


Figure 1. Physical map and transcriptional organization of the nif gene cluster of K. pneumoniae. The seven operons and the direction of transcription are indicated by the arrows above the physical map. Data were obtained from Roberts et al. (161) and Riedel et al. (157).

Table 2. *K. pneumoniae nif* gene products

Gene	Molecular Weight	Function
J	120,000	electron transport
H	35,000	dinitrogenase reductase subunit
D	56,000	α-subunit of dinitrogenase
K	60,000	β-subunit of dinitrogenase
Y	19 - 24,000	(-)
E	40 - 46,000	FeMo-co processing or insertion
N	50,000	FeMo-co processing or insertion
X	18,000	(-)
U	22 - 28,000	(-)
S	42 - 45,000	processing of *nif* H gene product
V	38 - 42,000	FeMo-co processing
M	27,000	processing of *nif* H gene product
F	10 - 22,000	electron transport
L	45 - 55,000	negative regulation (transcriptional)
A	57 - 66,000	positive regulation (transcriptional)
B	(-)	FeMo-co processing or insertion
Q	(-)	FeMo-co processing or insertion

Data were summarized from Roberts *et al.* (161) and Puhler and Klipp (150). (-) indicates that the molecular weight or function of this gene product is not known.

E, N, V, B, and Q and is inserted into dinitrogenase to form the active site of dinitrogen reduction (26.125,150,161,170). The reduction of dinitrogen to ammonium requires the presence of electron donors. *In vivo*, the electrons are transported to the dinitrogenase complex by the *nif* F and *nif* J gene products (161,170). The *nif* F protein has been identified as a flavodoxin (86,135,159), while the *nif* J protein is an iron-sulfur protein (19). At least three other *nif* genes, *nif* U, X, and Y, have been identified, but functions for these genes have not yet been established (4,34,150).

The regulation of the *nif* operon in *Klebsiella* is complex. Oxygen inactivates dinitrogenase as well as repressing expression of the *nif* genes (31,61,171). Ammonium (161) and temperatures above 37°C (83,214) also repress nitrogen fixation. Two genes, *nif* L and *nif* A, appear to exert regulatory control over the entire *nif* operon. The *nif* L protein is a negative regulatory factor and mediates oxygen repression and to a lesser extent ammonium repression (30,87,127). The *nif* A gene product is a positive regulatory factor and is required for expression of all of the *nif* operons except *nif* LA. The *nif* A protein is thermolabile (214), so *nif* gene expression is repressed at temperatures above 37°C.

Other proteins involved in the general nitrogen metabolism in the cell are also involved in the regulation of *nif* gene expression. The *gln* G (*ntr* C) and *gln* F (*ntr* A) gene products, which are required for the expression of the glutamine synthetase operon (*gln* ALG), are also required for positive activation of the *nif* operon (50.64). The activation of *nif* expression by *gln* G (*ntr* C) and *gln* F (*ntr* A) gene products is mediated at the *nif* LA operon, as is the repression of *nif* expression by *gln* L (*ntr* B) and *gln* G (*ntr* C) (50,58,64,142).

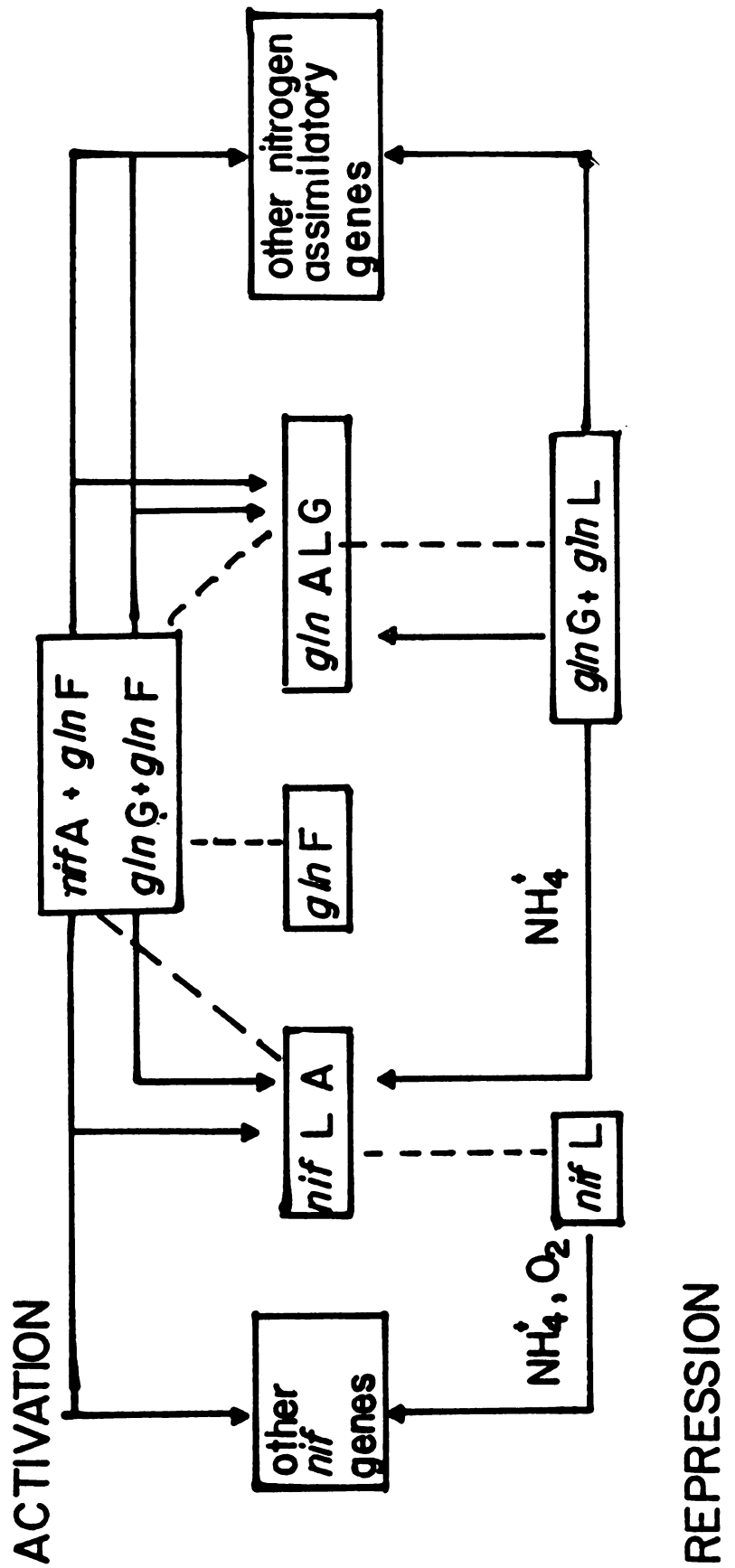
A model of nif regulation in K. pneumoniae is shown in Figure 2. In response to nitrogen starvation, the gln G (ntr C) gene product in concert with the gln F (ntr A) protein activates transcription of the nif LA operon (50,64). The products of the nif A and gln F (ntr A) genes are required for the expression of all other nif genes (58,142). In addition, the nif A gene product can substitute for the gln G (ntr C) protein in the activation of the gln ALG operon, other nitrogen assimilatory genes, and its own (nif LA) operon (58,142). The substitution is not reciprocal, however, since the K. pneumoniae gln G (ntr C) protein does not activate the K. pneumoniae nif H promoter and probably not promoters for the other nif genes as well (142).

Repression of nif expression in the presence of high (30 mM) ammonium is mediated at the nif LA operon by the gln L (ntr B) and gln G (ntr C) proteins. The nif L protein represses synthesis of the other nif proteins in the presence of oxygen and ammonium (30,87,127).

The regulation of nitrogen fixation in Rhizobium may occur by a somewhat different mechanism. Rhizobium strains are not repressed for dinitrogenase synthesis by "fixed" nitrogen compounds, such as ammonium (17). This might be expected since nitrogen-fixing bacteroids excrete ammonium into the plant cytosol.

Gene fusions constructed with the R. meliloti nif H promoter and the lac Z gene from E. coli show that the nif H promoter can be activated by the K. pneumoniae nif A protein (189). Sequencing data indicates about 50% homology between the nif H promoters for R. meliloti and K. pneumoniae, with several regions of exact homology (189). This suggests that R. meliloti may contain a regulatory protein analogous to the K. pneumoniae nif A protein. The gln G gene product may directly

Figure 2. A model of nif regulation in K. pneumoniae. gln G and gln F are positive transcriptional regulators of the general nitrogen metabolism. During nitrogen-limiting conditions of growth, gln G and gln F proteins activate the glutamine synthetase operon (gln ALG), the nif LA operon, and other nitrogen regulatory genes. The nif A protein can substitute for the gln G protein as an activator. In addition, the nif A protein activates transcription of the other nif genes. nif L is a negative transcriptional regulator of the other nif genes in the presence of oxygen or ammonium. gln L and gln G are also negative regulators during growth in the presence of ammonia.


other nitrogen assimilatory genes
gln ALG
glnG+ glnL
nifA + glnF
glnG+glnF
glnF
NH4+
nifLA
nifL
NH4+, O2
other nif genes
ACTIVATION
REPRESSION

regulate nif expression in some Rhizobium. The E. coli gln G (ntr C) gene product, acting in conjunction with the E. coli gln F (ntr A) activated the R. meliloti nif H promoter in nif H:lac Z gene fusions (142). In contrast, the K. pneumoniae gln G (ntr C) gene product cannot activate K. pneumoniae nif H transcription (142). The hypothesis that nif expression can be regulated by the gln G (ntr C) gene product is supported by sequence analysis of five promoters under the general nitrogen regulatory control of gln G and gln F gene products (143). These promoters, which included K. pneumoniae nif L, E. coli gln A, R. meliloti nif H and Salmonella typhimurium arg Tr and dhu A were found to have a 7 base pair consensus sequence (TTTTGCA) in the -15 region. The K. pneumoniae nif H promoter, which is not under gln G-gln F control has only partial homology (CCCTGCA) in this region.

Ruvkun and Ausubel (166) have shown that the K. pneumoniae nif genes are homologous to DNA from 19 other diverse nitrogen-fixing bacterial strains, and that the genes did not hybridize to DNA from 10 different non-nitrogen-fixing bacteria. The homologous region of nif DNA is localized to a 1.6 kb region of the nif H and nif D genes.

The interspecies homology of nif DNA has been useful in the isolation of nif DNA from gene libraries of R. meliloti and R. japonicum DNA (82,166). The availability of cloned nif DNA from Rhizobium has enabled preliminary studies of the genetic organization of the nif operon in Rhizobium to be carried out. In R. meliloti and R. leguminosarum, the nif H, D, and K genes are located adjacent to each other as in K. pneumoniae (5,40,143). However, the organization of the nif H, D, and K genes in R. japonicum apparently is different. Transcript mapping indicates that the nif D gene is adjacent to the nif K gene and that

the genes are transcribed together. The nif H gene is located a short distance upstream from nif DK, however, and is probably in another transcription unit (147).

The region of DNA adjacent to and containing the R. meliloti nif HDK genes has been analyzed using transposon Tn 5 insertion mutagenesis (39,40,168). The sites of insertion of Tn 5 have been physically mapped, and complementation analysis between genomic nif::Tn 5 insertions and nif::Tn 5 insertions on mobilizable cloning vectors has been carried out. Fix^- and Fix^+ phenotypes were observed on alfalfa plants inoculated with the Tn 5 insertion mutants. The Fix^- mutants were clustered in a 14 to 15 kb region of DNA, of which 1.9 kb was not essential for nitrogen fixation (39). This compares to a 24 kb segment of DNA which encodes the nif genes in K. pneumoniae (Figure 1). The presence of three transcriptional units in the 14 to 15 kb region of DNA containing the nif HDK genes were deduced from complementation analysis data (166). The nif HDK genes constitute one transcription unit about 6.3 kb long. The other two transcription units are located upstream from the nif HDK transcription start site. One of these transcriptional units is apparently transcribed in the opposite direction as compared to the nif HDK operon (39). These results indicate that the organization of the R. meliloti nif operon is different from that of K. pneumoniae where only one transcription unit, nif J is upstream from nif HDK (see Figure 1).

Plasmid DNA in Rhizobium. Historically, many investigators have reported that the nodulation (Nod) and nitrogen fixation (Fix) phenotypes are unstable (2,59,175). Prolonged storage of Rhizobium on certain media (117) or in sterilized soil (139,154) have resulted in up to

35% of the recovered colonies possessing a Fix$^-$ phenotype (192). Preliminary studies did not uncover a genetic basis for the instability of the symbiotic phenotypes (59).

More recently, Higashi (86) was able to demonstrate transfer of clover nodulation from R. trifolii to R. phaseoli. The clover nodulation phenotype could be partially eliminated by growth of the R. trifolii strain and the R. trifolii/R. phaseoli transconjugant in the presence of acridine orange. Several other reports have since been published showing the loss of nodulation ability from strains of Rhizobium as a result of growth in the presence of intercalating dyes (59,216). Intercalating dyes are known to eliminate some plasmids by interfering with their replication (38,88,91), suggesting that the genetic information coding for nodulation and nitrogen fixation may be located on plasmid DNA in Rhizobium.

Plasmids are extra chromosomal genetic elements present in a wide variety of gram-negative and gram-positive bacteria (38,136). Plasmids replicate autonomously (38) and exist in the cells as covalently closed circular (CCC) DNA molecules (183). Plasmids have a considerable variation in size and also in copy number, the number of plasmid molecules of one type stably maintained in a bacterial cell (38). A wide variety of functions are encoded on plasmid DNA, including: bacteriocin production, antibiotic resistance and promotion of conjugation (see 38,81,136 for Reviews).

Using techniques developed for the detection and isolation of plasmid DNA in other bacteria, a number of effective (Nod$^+$ Fix$^+$) and some ineffective (Nod$^+$ Fix$^-$) Rhizobium strains were analyzed for plasmid DNA. Although plasmids were not detected in all of the

strains examined, plasmids of molecular weight ranging from 5.5 x 10^6 to 64 x 10^6 were observed in R. "cowpea" (190), R. trifolii (106,195,217), and R. japonicum (104). No correlation could be made between nodulation and nitrogen fixation abilities and the presence or absence of plasmid DNA in these effective and ineffective strains of Rhizobium.

Agrobacterium, a genus of bacteria closely related to Rhizobium (69,80) causes tumorous growths, or crown galls, on dicotyledonous plants (57). The genes coding for tumor formation in Agrobacterium are encoded on large plasmids having molecular weights greater than 100 x 10^6 (57,198,203). The similarity between the formation of crown gall tumors by Agrobacterium and the induction of root nodules by Rhizobium led to the suggestion that large plasmids in Rhizobium may encode genes essential for the Rhizobium-legume symbiosis. Methods developed for the isolation of large plasmids from A. tumefaciens (41, 111,213) have been applied to Rhizobium. A wide range of large plasmids have been observed in R. leguminosarum (23,137,146), R. meliloti (35), and R. japonicum (72,123). Using these procedures, the isolation of plasmids greater than 200 Mdal was difficult and recovery was poor (11,23). However, new techniques (5,164,177), especially the "in gel" lysis technique of Ekhardt (63), allow the detection and isolation of very large plasmids. Indeed, plasmids much greater than 300 Mdal in size, termed "megaplasmids", have been detected in virtually all strains of R. meliloti using these techniques (5,164).

The number and size of the plasmids in Rhizobium varies greatly (11). The diversity of plasmids in Rhizobium can be seen in the Ekhardt agarose gel shown in Figure 3. As many as seven plasmids may

be present in a strain (Figure 3, lane c). Plasmids of molecular weight ranging from about 30 x 10^6 to greater than 325 x 10^6 can be detected in Rhizobium.

Genetic Functions Encoded on Plasmids in Rhizobum. In Rhizobium, plasmid DNA can account for 25 to 30% of the total DNA due to the number and large size of the plasmids (11). However, few genetic functions have been determined to be specified on these plasmids.

Bacteriocins, compounds which inhibit the growth of closely related species of bacteria, are known to be plasmid encoded in enterobacteria (76). Production of bacteriocins by Rhizobium has been described by many investigators (165,176,178) but was not known to be plasmid encoded.

Hirsch (89) identified three transmissible plasmids in R. leguminosarum which code for a bacteriocin. The bacteriocin was classified as medium in size since it diffused about 5 mm in agar plates but could not diffuse through a dialysis membrane. A second locus on these plasmids repressed the synthesis of a small bacteriocin when the plasmids were transferred into R. leguminosarum strains which normally produce the small bacteriocin.

Pea nodulation ability was found to cotransfer at high frequency (10^{-3} to 10^{-2}) with the ability to produce the medium bacteriocin (22,100). Transfer of pR11JI, a conjugative plasmid in R. leguminosarum which codes for the production of medium bacteriocin production, into Nod^- strains of R. leguminosarum resulted in transconjugants with a Nod^+ Fix^+ phenotype on pea plants (100). Transfer of mutant (Fix^-) derivatives of pR11JI (29) into a Nod^- strain of R. leguminosarum containing a deletion in one of its plasmids resulted

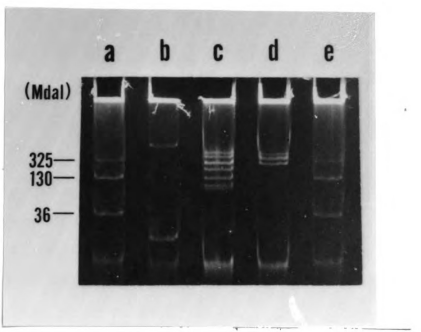


Figure 3. Ekhardt agarose gel electrophoresis of plasmid DNA in A. tumefaciens and three strains of Rhizobium. Lanes a and e, A. tumefaciens strain C58(RP4); b, R. meliloti strain Su27; c, R. leguminosarum strain T69; d, R. trifolii strain 0403. Samples were prepared and Ekhardt agarose gel electrophoresis performed by the author as described in Chapter II Methods.

in transconjugants with Nod$^+$ Fix$^-$ phenotypes (22). This indicates that both *nod* and *nif* genes were contained on the region of deleted plasmid DNA. Similar results have been obtained in other species of *Rhizobium*. Transfer of a *R. trifolii* plasmid from Nod$^+$ strains of *R. trifolii* to Nod$^-$ strains conferred clover nodulation ability on the recipient (95,179,215). Deletion of plasmid DNA in *R. meliloti* (5,164) and the elimination of a 111 Mdal *R. leguminosarum* plasmid (146) resulted in a Nod$^-$ phenotype on the respective host plant.

The *nif* structural genes have been located on large plasmids in many *Rhizobium* strains (5,79,94,123,138,164). A heterologous *nif* probe from *K. pneumoniae* hybridized to a Southern filter of restriction endonuclease-digested total plasmid DNA from *R. leguminosarum* (138). Results from similar Southern hybridization filter analyses have indicated that one of the plasmids in strains of *R. trifolii* and *R. phaseoli* encode the *nif* structural genes (147).

The specific plasmid which encodes the *nif* genes has since been identified in some *Rhizobium* strains. Hybridization of a heterologous *nif* probe to a Southern filter of plasmids separated on agarose gels has identified the *nif*-containing plasmid in *R. leguminosarum* (94,148), *R. phaseoli* (94), and *R. japonicum* (79,123).

Strains of *R. meliloti* harbor very large plasmids ("megaplasmids") coding for genes required for nodulation of alfalfa (7,164). Hybridization with cloned *R. meliloti nif* DNA has shown that the *nif* structural genes are also located on this plasmid (5,164). Using a series of overlapping cosmid clones of *R. meliloti* DNA, Long *et al.* (116) have localized a nodulation gene with 30 kb of the *nif* structural genes.

Hombrecher et al. (77) have reported linkage of nif and nod genes on plasmids in R. leguminosarum and R. phaseoli.

Genetic determinants for a hydrogen uptake enzyme (Hup) have been shown to be plasmid-encoded in R. leguminosarum (24). The uptake hydrogenase permits recycling of hydrogen gas, a by-product of the dinitrogenase-catalyzed reduction of dinitrogen (174). Nodulation ability (Nod^+) was transferred from a Hup^+ R. leguminosarum strain to a Nod^- Hup^- strain using a kanamycin-resistant derivative of a plasmid (pRl3JI) which is known to mobilize other plasmids (22). Approximately 70% of the kanamycin-resistant transconjugants were Nod^+ Hup^+, suggesting nod and hup genes are linked on a plasmid in this R. leguminosarum strain.

The expression of plasmid DNA in the bacteroid state has also been analyzed (191,202,203). Krol et al. (107) have obtained hybridization of R. leguminosarum plasmid DNA to RNA from bacteroids. Little or no hybridization was observed to RNA from vegetative bacteria. The RNA hybridized to only one of the two plasmids present in the R. leguminosarum strain (108). Regions of DNA of a different R. leguminosarum plasmid which is heavily transcribed in bacteroids have been mapped (148). One of the regions contains the nif structural genes. The functions of the genes in the other transcribed areas are at present unknown, but presumably are involved in the maintenance of nitrogen-fixing nodules.

Other genetic functions which may be involved in the establishment and maintenance of the symbiosis have been determined to be plasmid-encoded. A plasmid in a R. leguminosarum strain appears to code for genes required for synthesis of exopolysaccharide (146). Isolates

which had lost a 111 Mdal plasmid as a result of "heat-curing", had a rough colony morphology, indicative of an inability to synthesize exopolysaccharide. These isolates also had altered phage sensitivities and were unable to nodulate pea plants.

The ability of strains of R. meliloti to produce polygalacturonase has been linked to the presence of a 59.6 Mdal plasmid (141). Curing of the plasmid by growth of R. meliloti strains in the presence of acridine resulted in isolates with low levels of polygalacturonase activity. The role of this enzyme in the degradation of the root hair cell wall during the infection process is still in dispute (6,43,44).

CHAPTER I

Preliminary Studies on the Presence of Plasmid DNA and Plasmid-Coding of Genes for Nodulation and Nitrogen Fixation in *Rhizobium*.

INTRODUCTION

At the time this research was initiated, plasmid coding of *sym* genes had been implicated in strains of *Rhizobium*. Treatment of strains of *Rhizobium* with plasmid "curing" agents, such as acridine dyes (85,216), or culturing *Rhizobium* under certain growth conditions resulted in the loss of nodulation ability (59,175,187). Conjugal transfer of clover nodulation and nitrogen fixation (*nif*) genes was observed, and was suggested to be plasmid-mediated (60,85). However, no direct correlation of the absence or transfer of a specific plasmid with the loss or transfer of symbiotic properties had been reported at that time.

Two widely used plasmid isolation techniques, the SDS-salt precipitation technique (73) and the cleared-lysate technique (37) are suitable for the isolation of plasmids of molecular weight <100 Mdal. However, these techniques were not suitable for the detection of plasmids in most strains of *Rhizobium*. Subsequently, techniques for the isolation of large (>100 Mdal) plasmids have been developed (35,41,111).

In this Chapter, I report and discuss: 1) two methods for the isolation and detection of plasmids in strains of *Rhizobium*; 2) attempts to eliminate or "cure" plasmids from strains of *Rhizobium* and the examination of isolates from the treated cultures for the loss of symbiotic properties.

MATERIALS AND METHODS

Materials. Yeast extract and Bacto agar were obtained from Difco Laboratories, Detroit, MI. Acridine orange, ethidium bromide, cytochrome c (Type III), pronase (Type XIV) and Dowex 50 were purchased from Sigma Chemical Company, St. Louis, MO. Sodium N-laurylsarcosinate (sarkosyl) was obtained from ICN Pharmaceuticals, Plainview, NY. Sodium dodecyl sulfate (SDS) was purchased from either Pierce Chemical Company, Rockford, IL, or Bio-Rad Laboratories, Richmond, CA. Sucrose was obtained from Schwartz/Mann, Orangeburg, NY. Agarose was purchased from Bio-Rad Laboratories. ^{3}H-Thymidine (2 Ci/mmole) was obtained from New England Nuclear, Boston, MA. Platinum:paladium (80:20; 0.008 inch diameter) was obtained from Ted Pella, Inc., Tustin, CA. Scintillation fluid contained 66.7% toluene, 33.3% triton, 0.5% PPO, 0.01% POPOP (v:v:w:w). Photography of ethidium bromide-stained gels was carried out using Polaroid Type 57 or Type 667 film (Polaroid Corporation, Cambridge, MA).

Bacterial strains. The strains of Rhizobium used were: R. leguminosarum strain 128C53, obtained from J. Burton, The Nitragin Company, Milwaukee, WI; R. trifolii strain T37, obtained from F.B. Dazzo, Michigan State University, East Lansing, MI; R. "cowpea" strain CB756, obtained from J. Tjepkema, University of Maine, Orono, ME; R. "cowpea" strain 32H1, obtained from J.M. Vincent, University of Australia, Sydney, Australia; and Agrobacterium tumefaciens strain

C58(RP4) obtained from T.C. Currier, Kansas State University, Manhattan, KS.

All strains of Rhizobium were symbiotically effective on their normal host plant. R. trifolii strain T37 (118) as well as R. "cowpea" strains CB756 and 32H1 (144) have been reported to fix nitrogen in culture. A. tumefaciens strain C58(RP4) contains the 36 Mdal broad-host-range plasmid RP4 (52), a 130 Mdal tumor-inducing (Ti) plasmid (93), and a 325 Mdal cryptic plasmid (35).

Media. Bacteria were routinely grown on yeast extract-mannitol (YEM) medium containing (per liter redistilled water): 10.0 g mannitol, 2.0 g yeast extract, 0.2 g KH_2PO_4, 0.3 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.1 g NaCl. YEM medium was solidified with 1.5% Bacto agar. Bacteria grown for plasmid isolations were cultured in YE_2 medium, which was similar to YEM but contained no mannitol. Bacterial DNA was labeled by culturing bacteria in Wright's medium (195) supplemented with 0.1 mCi/ml ^{3}H-thymidine (2 Ci/mmole). Wright's medium contained: 0.2 g NaCl, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaSO_4 \cdot 7H_2O$, 0.1 g $CaCO_3$, and 1.0 g yeast extract per liter redistilled water. Bacteria to be assayed for dinitrogenase activity (acetylene reduction) in culture were grown on CS7 medium (144). CS7 medium was prepared by mixing 0.1 volume of Solution A and 0.01 volume of Solution B with 0.8 volumes of redistilled water. Agar (1 g/l) was added and the medium was sterilized by autoclaving. The autoclaved medium was cooled to 50°C and 0.1 volume of filter sterilized Solution C was added. The CS7 medium was dispensed into 3-dram vials. Solution A contained: 0.3 g KH_2PO_4, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.07 g KCl, 0.035 g $MgSO_4 \cdot 7H_2O$, 1.0 g

myo-inositol, and 2.55 g succinic acid in 100 ml of redistilled water. Solution B contained: 98 mg $MnSO_4 \cdot H_2O$, 50 mg H_3BO_3, 10 mg $ZnSO_4 \cdot 7H_2O$, 10 mg KI, 2 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg $Na_2MoO_4 \cdot 2H_2O$, 1.0 mg $CoCl_2 \cdot 6H_2O$, and 201 mg disodium EDTA per 100 ml of redistilled water. Solution C contained 9.38 g arabinose, 731 mg glutamine, 37.5 mg $FeSO_4 \cdot 7H_2O$, 13 mg thiamine·HCl, 13 mg nicotinic acid and 1.2 mg pyridoxine HCl per 100 ml of redistilled water. The pH of the medium was adjusted to pH 5.9.

Currier-Nester Plasmid Isolation Procedure. This procedure is suitable for the isolation of large ($\leq$130 Mdal) plasmids (41). Cells were grown in YE_2 medium to late log phase (150-175 Klett units; 1 to 2 x 10^9 cells/ml). Bacteria were pelleted by centrifugation at 10,000 x g for 20 min at 4°C, washed once with 10 mM Tris·HCl (pH 8.0), 1 mM EDTA (TE buffer) and resuspended in 0.2 volumes TE buffer. Pronase (10 mg/ml predigested at 37°C for 1.5 h) and SDS (10%) were added to a final concentration of 0.5 mg/ml and 1%, respectively. The cells were incubated for 45 min at 37°C. The viscosity of the lysate was reduced by shearing the DNA for 1 min with a vortexer (Scientific Products Model S8220) or with a Serval Omni Mixer (for volumes > 100 ml). The sheared lysate was adjusted to pH 12.4 with 3 M NaOH and incubated at room temperature for 10 min with occasional stirring. The lysate was neutralized with 2 M Tris·HCl (pH 7.0) to pH 8.5-9.0 and stirred gently for 3 min. Sodium chloride (30%; w/v) was added to the neutralized lysate to a final concentration of 3% (w/v) NaCl and the lysate was extracted with an equal volume of redistilled phenol equilibrated with 3% (w/v) NaCl. The phenol and aqueous phases were separated by centrifugation at 6000 x g for 10 min at 4°C. Plasmid DNA was

contained in the aqueous phase while the denatured chromosomal DNA banded at the interface. The aqueous phase was recovered using an inverted 5 ml pipet and was extracted with an equal volume of chloroform:isoamyl alcohol (24:1). The aqueous phase was recovered as before, and 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol were added to precipitate the DNA. After a minimum of 4 h at -20°C, the DNA was pelleted by centrifugation at 12,000 x g for 20 min at 4°C. The DNA pellet was dried and was resuspended in 200-500 µl TE buffer. Samples were analyzed for plasmid DNA by agarose gel electrophoresis or CsCl·EtBr density equilibrium centrifugation.

Alkaline Lysis Procedure for Plasmid Isolation. This method (35) utilizes a non-enzymatic and more gentle lysis than the Currier-Nester procedure. Bacteria were grown in YE_2 medium to a density of about 50 Klett (5×10^8 cells/ml). Cells were pelleted by centrifugation at 12,000 x g for 10 min at 4°C, washed first with 1 M NaCl, 10 mM EDTA, and then washed with TE buffer. The cells were resuspended in TE buffer at a concentration of 0.2 g cells/ml. The cell suspension (0.5 ml) was added to 9.5 ml alkaline lysis buffer (1% (w/v) Sarkosyl in TE buffer, pH 12.45). A 2.5 cm stir bar was added and the mixture was stirred for 90 sec at 100 rpm. The mixture was incubated at 34°C for 20 min, and neutralized with 0.6 ml 2 M Tris·HCl (pH 7.0), and was stirred for 2 min at 100 rpm. Sodium chloride (0.1 volume of a 30% (w/v) solution) was added and the solution was stirred another 10 sec. The lysate was incubated at room temperature for 30 min. An equal volume of redistilled phenol equilibrated with 3% (w/v) NaCl was added. The mixture was stirred rapidly (about 300 rpm) for 10 sec, and then the stirring rate was decreased to 100 rpm for 2 min. The two phases

were separated by centrifugation at 5000 x g for 10 min at 4°C. The clear upper aqueous phase was transferred to a new tube using an inverted 5 ml pipet. The plasmid solution was brought to 0.3 M sodium acetate with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol were added. DNA was precipitated at -20°C and pelleted by centrifugation at 12,000 x g for 20 min at 4°C. The supernatant fluid was decanted and the DNA pellet was allowed to dry. The pellet was resuspended in 100 µl TE buffer. Samples were analyzed for plasmid DNA by agarose gel electrophoresis or further purified by CsCl·EtBr density equilibrium centrifugation. The procedure can be scaled up 5-fold for the isolation of greater amounts of plasmid DNA.

[^{3}H]Thymidine Labeling of Bacterial DNA. The inocula for [^{3}H]thymidine labeling studies were grown in YEM medium to late log phase (1 to 2 x 10^9 cells/ml; 150-175 Klett units). An aliquot (0.1 ml) was used to inoculate 10 ml of Wright's medium containing 10 µCi/ml [^{3}H]thymidine (2 Ci/mmole). The cultures were grown for 24 h at 30°C with shaking. The culture labeled with [^{3}H]thymidine was mixed with a 40 ml of unlabeled culture. Cells were pelleted by centrifugation at 12,000 x g for 10 min at 4°C and washed once with 1 M NaCl, 1 mM EDTA and once with TE buffer. The cells were resuspended at a concentration of 0.2 g cell/ml and lysed using the alkaline lysis procedure. Analysis for plasmid DNA was carried out on sucrose gradients.

Sucrose Gradient Centrifugation. Neutral and alkaline linear 5-20% sucrose gradients were used to analyze ^{3}H-labeled bacterial DNA for plasmids (111,213). DNA samples to be analyzed on neutral sucrose gradients were isolated by the alkaline lysis procedure. Samples were layered on linear 5-20% neutral sucrose gradients (10 ml) prepared in

100 mM NaCl, 10 mM Tris·HCl (pH 8.0), 1 mM EDTA (STE buffer). The gradient was centrifuged at 37,000 rpm for 60 min at 4°C in a Beckman SW41 rotor. Fractions (100 µl) were collected from the bottom directly into scintillation vials. Redistilled water (0.5 ml) and 5 ml of scintillation fluid (see Materials) were added and radioactivity was determined by liquid scintillation spectrometry. Samples to be analyzed on alkaline sucrose gradients were lysed as described in the alkaline lysis procedure, but were not neutralized. Instead, lysates were sheared for 30 sec (two-15 sec pulses) using a vortexer (at full speed) and 1.0 ml of the lysate was layered on an alkaline sucrose gradient. Linear 5-20% alkaline sucrose gradients (10 ml) were prepared in 1 M NaCl, 0.3 M NaOH, 10 mM Tris·HCl (pH 8.0), 50 mM EDTA. The gradient was centrifuged at 37,000 x g for 20 min at 4°C in a Beckman SW41 rotor. Fractions were collected and radioactivity was determined as described for neutral sucrose gradients.

Agarose Gel Electrophoresis. Plasmid DNA was separated on vertical 0.7% agarose gels (14 mm x 12 mm x 3 mm). Tris-borate - EDTA (TBE) electrophoresis buffer consisted of 89 mM Tris (Trizmabase), 89 mM H_3BO_3 and 8.9 mM EDTA (129). Electrophoresis was carried out at 100 volts for 3 h. The gel was stained for 15 min with ethidium bromide (0.5 µg/ml). DNA was visualized with UV light and the gel was photographed.

CsCl·EtBr Density Equilibrium Centrifugation. Supercoiled (CCC) plasmid DNA was separated from nicked plasmid and linear chromosomal DNA by CsCl·EtBr density equilibrium centrifugation (152). Cesium chloride (9 g) was dissolved in 9 ml TE buffer. The resuspended plasmid DNA in 1 ml of TE buffer and 0.3 ml of ethidium bromide (10 mg/ml)

were added to the CsCl solution and the solutions were gently mixed to avoid shearing the DNA. The gradients were centrifuged at 35,000 rpm for 36 h at 4°C in a Beckman Ti 50.1 rotor. The plasmid DNA was visualized with UV light and removed from the gradient. Cesium chloride was removed from the plasmid DNA solution by dialysis against one 1 TE buffer (4 changes). Ethidium bromide was removed from the DNA by Dowex-50 chromatography in TE buffer (152) or by repeated extraction with isopropyl alcohol. Plasmid DNA was concentrated by ethanol precipitation at -20°C. The concentration of plasmid DNA was determined by the absorbance at 260 nm.

Electron Microscopy of Plasmid DNA. The microspreading modification (98) of the protein monolayer technique of Kleinschmidt (105) was used to prepare DNA samples for electron microscopy. Hyperphase solutions (10 µl) containing 1 µg DNA, 1 µg cytochrome c, and 0.5 M ammonium acetate were spread down a glass rod onto approximately 2 ml of hypophase solution (0.25 M ammonium acetate). The protein monolayer and plasmid DNA were picked up with Parlodion- coated, 200-mesh copper grids. The DNA was stained (205) for 30 seconds with a 1:100 dilution of a solution of 5 mM uranyl acetate, 5 mM HCl in 95% ethanol, and rotary shadowed with platinum:paladium (80:20). Electron microscopy was carried out on a Siemens Elmiskop 1A electron microscope. Magnification was calibrated using a carbon replica of a diffraction grating (54,864 lines/inch). Contour length measurements of DNA molecules were determined from about 3X print enlargements using a Keufel and Esser opisometer. The print magnification was determined from the ratio of the distance between two objects on the print to the corresponding distance on the negative. Molecular weights were calculated from contour

length measurements assuming a mass to length ratio of 2.07 Mdal/μm (38).

Acetylene Reduction Assay on Rhizobium in Culture. Dinitrogenase activity was assayed by measuring the production of ethylene from acetylene (78). Strains to be tested for dinitrogenase activity in culture were grown in YEM medium to late log phase. Three loops of the culture were stabbed into 2.5 ml of CS7 medium solidified with 1% agar in a three-dram vial. Three replicates of each strain were inoculated. The vials were incubated at 30°C for 7 days. The screw caps were removed aseptically and the vials were sealed with sterile serum caps. Air (1 ml) was removed and 1 ml of acetylene (generated from calcium carbide and water) was added. The vials were incubated another 2 days at 30°C, at which time 0.2 ml samples of gas were removed. The gas samples were analyzed for ethylene on a gas chromatograph (Varian Model 3700) equipped with a Porapak N column (2 mm x 2 m).

Growth of R. "cowpea" strain 32H1 at Elevated Temperature. R. "cowpea" strain 32H1 was grown in two - 100 ml cultures of YEM medium at 30°C with shaking. When the cell density reached 30 Klett units (about 2 x 10^8 cells/ml), one culture was shifted to 37°C and both cultures were incubated for another 12 days. Suitable 10-fold serial dilutions of the culture incubated at 37°C were plated on YEM agar plates to follow cell viability. At the end of 12 days, suitable 10-fold serial dilutions of heat-treated (37°C growth) culture were plated on YEM agar. Fifty colonies were randomly chosen and tested for the ability to fix nitrogen in culture.

Acridine Orange Treatment. Bacteria were grown in YEM medium for 3 days at 30°C. An aliquot of the culture was diluted to give a cell

density of about 5 x 10^6 cells/ml and 1 ml of the diluted culture was transferred to flasks containing 50 ml of YEM medium supplemented with 0, 5, 10, or 20 µg/ml acridine orange. The cultures were incubated for 24 h at 30°C with shaking and then 10-fold serial dilutions were plated on YEM agar. Plates were incubated at 30°C and single colonies were randomly chosen. The isolates were tested for the ability to reduce acetylene in culture and to nodulate the appropriate host plant.

Nodulation Test. The symbiotic properties of R. trifolii strain T37 isolates were examined by inoculating white clover (Trifolium repens var. Ladino). Clover seeds were surface-sterilized by washing with 70% ethanol for 30 sec followed by a 5 min treatment with 0.2% $HgCl_2$ acidified with 5 ml 12 N HCl per liter. The seeds were rinsed eight times with sterile redistilled water and germinated in the dark at 20°C on water agar plates. Two seedlings were transferred aseptically to each 18 x 150 mm culture tube containing 9 ml of Jensen medium agar (201). Alternatively, the surface-sterilized clover seeds were germinated directly on the Jensen agar slant. Jensen medium contained (per liter redistilled water): 1.0 g $CaHPO_4$, 0.2 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl and 0.1 g $FeCl_3$. Bacteria to be used as an inoculum were grown to late log phase (1 to 2 x 10^9 cells/ml; 150-175 Klett units) in YEM medium. An aliquot (0.1 ml) of the culture was pipeted directly onto the seedlings. The plants were placed in a growth chamber at 20°C with a 16 h photoperiod. After 5 weeks, plants were scored for root nodules and were tested for acetylene reduction activity.

The symbiotic properties of isolates of R. "cowpea" strain 32H1 were examined on cowpea (Vigna unguiculata L). Cowpea seeds were

surface-sterilized as described for clover seeds and were germinated directly in pots (10 cm) containing Perlite. Plants were inoculated with 5 ml cultures of isolates of R. "cowpea" strain 32H1 treated with acridine orange. The plants were grown in a greenhouse and were maintained on nitrogen-free nutrient solution (66). The photoperiod was extended to 16 h with supplemental fluorescent lighting (200 $\mu E/m^2$). After 4 weeks, plants were examined for root nodules and were tested for acetylene reduction activity.

Acetylene Reduction Assay on Nodulated Plants. Tubes containing clover plants were sealed with serum caps and 0.1 volume (2 ml) of air was removed and replaced with an equal volume of acetylene. After 15 min at room temperature, 0.2 ml samples were removed and analyzed for ethylene on a gas chromatograph.

Cowpea plants were removed from the pots and the shoot was excised. The roots were placed in a 250-ml Erlenmeyer flask. The flask was sealed with a serum cap and 0.1 volume (25 ml) of air was removed and replaced with an equal volume of acetylene. Gas samples (0.5 ml) were taken after 15 min and analyzed for ethylene on a gas chromatograph.

RESULTS AND DISCUSSION

Isolation of Plasmid DNA. Two procedures were used for the isolation of plasmid DNA from Rhizobium and Agrobacterium: the Currier-Nester procedure and the alkaline lysis procedure. The Currier-Nester procedure employed a SDS-pronase lysis, followed by a brief shearing step to reduce the viscosity and facilitate the denaturation of the DNA. The alkaline lysis procedure used included several modifications which increased the yield of plasmid DNA and also allowed the isolation of larger plasmids. The bacteria were lysed non-enzymatically at high pH, thus accomplishing the cell lysis and denaturation of the DNA in one step. The lysis of cells with pronase (which may be contaminated with nucleases) and the DNA shearing step found in the Currier-Nester procedure were not utilized in the alkaline lysis procedure. The rate of stirring during the cell lysis and subsequent steps was also carefully controlled at about 100 rpm to minimize shearing of plasmid DNA. Using these procedures, plasmid DNA of 36 to about 325 Mdal could be detected in the plasmid preparations after: 1) sucrose gradient centrifugation; 2) agarose gel electrophoresis; 3) CsCl·EtBr density equilibrium centrifugation; and 4) electron microscopy.

Sucrose Gradient Centrifugation. In alkaline environments, supercoiled plasmid molecules collapse into condensed structures with sedimentation coefficients 3 to 4 times those of similarly-sized linear DNA (156). This allows plasmid DNA to be separated from chromosomal DNA on

alkaline sucrose gradients. Strains of Rhizobium were cultured in the presence of [^{3}H]thymidine and lysed using the alkaline lysis procedure. Lysates were layered onto alkaline sucrose gradients and the gradients centrifuged to separate plasmid DNA from chromosomal DNA. Gradients were fractionated and the amount of radioactivity contained in each fraction was determined. A peak of ^{3}H-labeled plasmid DNA (fractions 24-26) which sedimented faster than the chromosomal DNA was observed in the sedimentation profile of an alkaline sucrose gradient of R. leguminosarum strain 128C53 DNA (Figure 4). A similar peak of radioactivity was not observed in alkaline lysates of ^{3}H-labeled DNA from R. "cowpea" strain 32H1 (Figure 4) or strain CB756 (data not shown).

Plasmid DNA can also be separated from chromosomal DNA by centrifugation through neutral sucrose gradients. R. trifolii strain T37 was cultured in the presence of [^{3}H]thymidine. The cells were lysed using the alkaline lysis procedure and the lysate neutralized. The lysate was layered on a linear 5 - 20% neutral sucrose gradient and centrifuged. The sedimentation profile of a neutral lysate of ^{3}H-labeled R. trifolii strain T37 is shown in Figure 5. The shoulder of radioactivity in fractions 29-34 contained supercoiled plasmid DNA.

The detection of plasmids on sucrose gradients was hampered by the low incorporation of label into bacterial DNA. In addition, the cell lysates were briefly sheared in order to reduce the viscosity of the lysates which probably resulted in the loss of some plasmid DNA.

The size and number of plasmids could not be readily determined from the gradient profile of strains in which plasmids were detected. A single peak of radioactivity corresponding to plasmid DNA was

Figure 4. Alkaline sucrose gradient sedimentation profile of [^{3}H]thymidine-labeled DNA from cells lysed using the alkaline lysis procedure. Gradients were fractionated from the bottom, 100 µl fractions were collected and radioactivity was determined. (0), R. leguminosarum strain 128C53; ([]), R. "cowpea" strain 32H1.

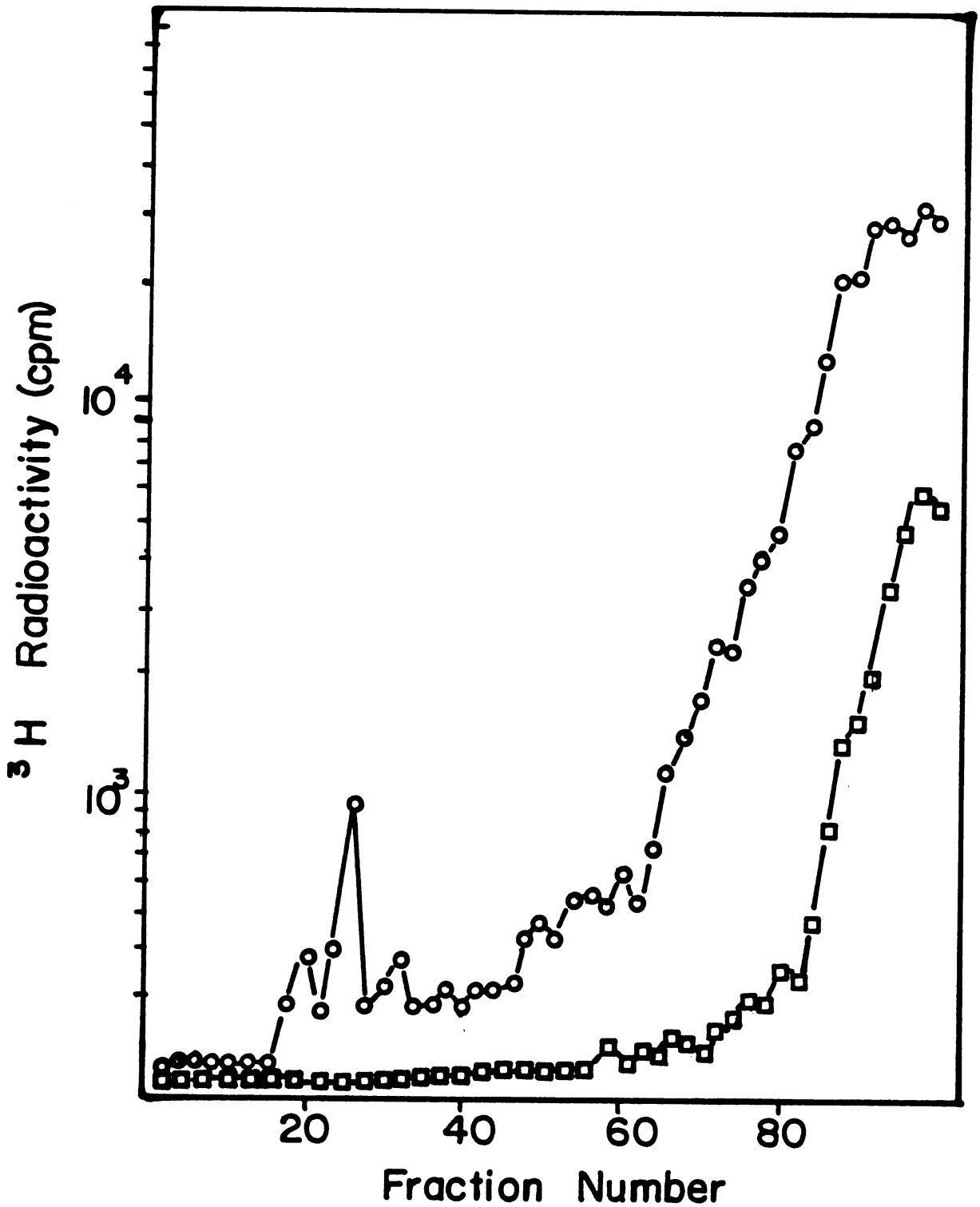
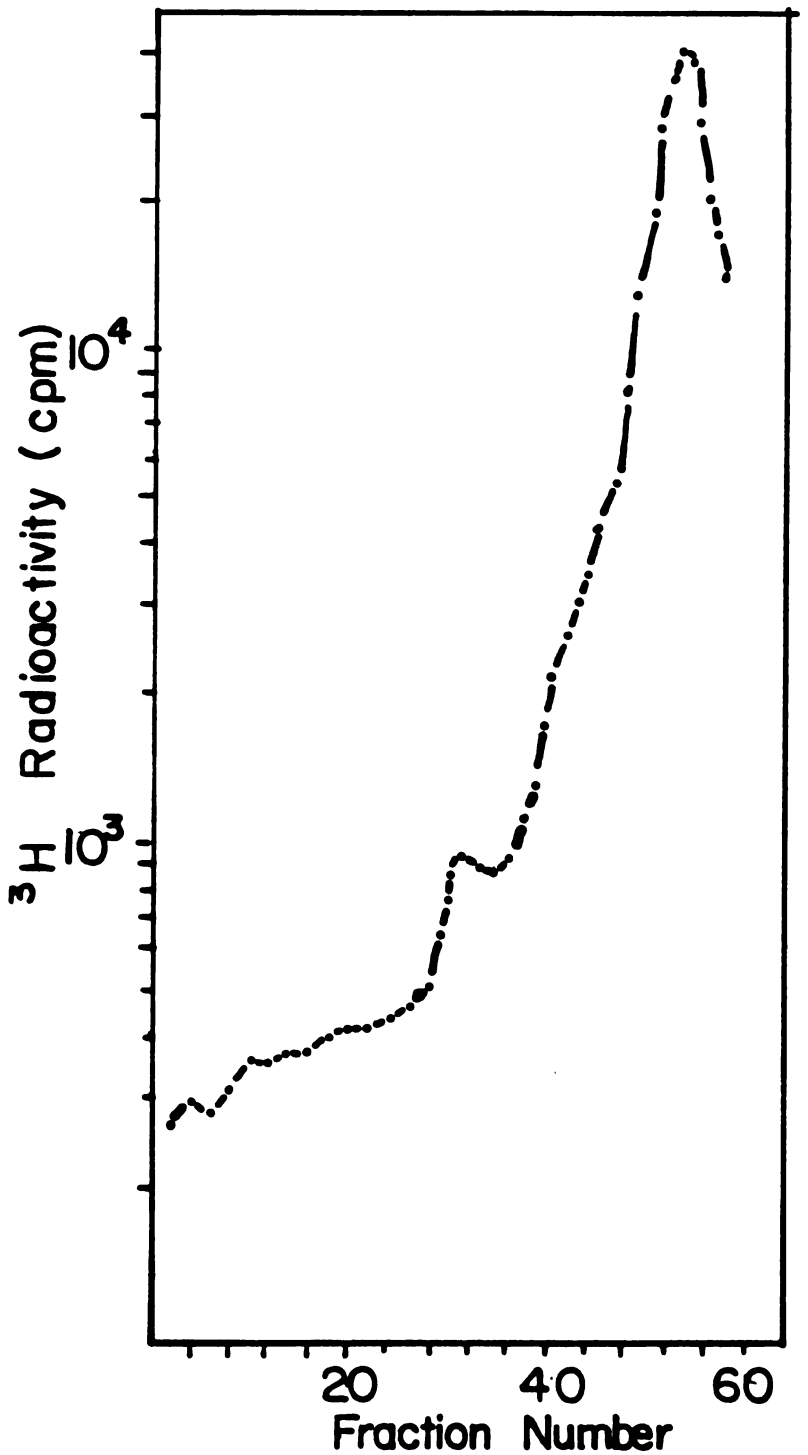

^{3}H Radioactivity (cpm)
10^4
10^3
20
40
60
80
Fraction Number

Figure 5. Neutral sucrose gradient sedimentation profile of [^{3}H]thymidine labeled cell lysate of R. trifolii strain T37. Cells were labeled as described in Methods and lysed using the alkaline lysis procedure. The lysate was neutralized and layered on a linear 5 - 20% neutral sucrose gradient. The gradient was centrifuged, fractions (100 µl) were collected and radioactivity was determined as described in the Methods.


^{3}H Radioactivity (cpm)
10^4
10^3
20
40
60
Fraction Number

detected in sucrose gradients of R. leguminosarum strain 128C58 (Figure 4) and R. trifolii strain T37 (Figure 5). R. trifolii strain T37 contains at least three plasmids (see Chapter II) as does R. leguminosarum strain 128C53 (Figure 6). Therefore, while sucrose gradient centrifugation can be used to confirm the presence of plasmid DNA in Rhizobium, the size and number of plasmids can not be determined.

Agarose Gel Electrophoresis of Plasmid DNA. Agarose gel electrophoresis was used successfully to identify two plasmids in crude extracts of plasmid DNA from A. tumefaciens strain C58(RP4) using the Currier-Nester plasmid isolation procedure (Figure 6, lane 1). Plasmid DNA could not be detected in R. "cowpea" strain 32H1 or strain CB756, or in R. trifolii strain T37 by agarose gel electrophoresis of plasmid preparations from these strains using the Currier-Nester procedure. Plasmid DNA from R. trifolii strain T37, however, was detected on CsCl·EtBr gradients using this procedure.

A third, high molecular weight plasmid was detected in A. tumefaciens strain C58(RP4) using the more gentle alkaline lysis procedure. The estimated molecular weight of this plasmid, pAtC58, was about 325 x 10^6 (Figure 6, lane 2). Freeze-thawing of the A. tumefaciens strain C58(RP4) plasmid sample resulted in the loss of pAtC58 (Figure 6, lane 3). Using the alkaline lysis procedure, two large plasmids were detected in R. trifolii strain T37 and three plasmids were detected in R. leguminosarum strain 128C53. As with the Currier-Nester procedure, repeated attempts to detect or isolate plasmid DNA in the R. "cowpea" strains using the alkaline lysis procedure were unsuccessful. This apparent absence of plasmids in the two R. "cowpea" strains may reflect the fact that these two strains do not

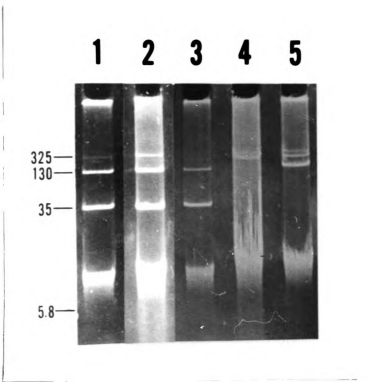


Figure 6. Agarose gel electrophoresis of plasmid DNA. Lanes 1-3, A. tumefaciens strain C58(RP4); lane 4, R. trifolii strain T37; lane 5, R. leguminosarum strain 128C53. The plasmid DNA in lane 1 was isolated by the Currier-Nester procedure. Samples in lanes 2-5 were isolated using the alkaline lysis procedure. Plasmid DNA in lane 3 was subjected to several freeze-thaw cycles. Molecular weight estimates were obtained from the relative mobility of plasmids of known size in agarose gels: pBR313, 5.8 Mdal; RP4, 35 Mdal; pTiC58, 130 Mdal; pAtC58, 325 Mdal.

contain plasmids. However, this does not seem likely, since Nuti et al. (137) have detected plasmid DNA in a strain of R. "cowpea". A more likely possibility is that these strains contain large plasmids which are not detected using the alkaline lysis procedure. Plasmids were not detected in several strains of R. meliloti using this procedure (146). Subsequent analysis of the R. meliloti strains using the Ekhardt agarose gel technique (discussed in Chapter II) resulted in the detection of extremely large plasmids (>>300 Mdal).

CsCl·EtBr Density Equilibrium Centrifugation. Plasmid DNA was separated from chromosomal DNA on CsCl·EtBr density equilibrium gradients. Less intercalating dye is bound by intact, CCC plasmids than by linear DNA, resulting in a smaller decrease in the density of the supercoiled plasmid-dye complex (152). Supercoiled plasmid DNA was isolated from R. leguminosarum strain 128C53 and A. tumefaciens strain C58(RP4) using the Currier-Nester procedure and the alkaline lysis procedure (Figure 7). A very faint CCC plasmid band was also observed for R. trifolii strain T37 using the Currier-Nester procedure. A more intense plasmid band reflecting a higher concentration of plasmid DNA was observed with R. trifolii strain T37 plasmid preparations obtained using the alkaline lysis procedure. Supercoiled plasmid DNA was observed in CsCl·EtBr gradients of R. "cowpea" strain 32H1 and strain CB756 DNA using either plasmid isolation procedure.

CsCl·EtBr density equilibrium centrifugation is useful both for the detection of plasmid DNA in Rhizobium and for the purification of plasmid DNA. Two major limitations are apparent with this technique. First, no information is obtained regarding the size and number of plasmids present. Only a more dense band corresponding to supercoiled

Figure 7. CsCl·EtBr density equilibrium gradient of plasmid DNA isolated by the alkaline lysis procedure. (A) R. leguminosarum strain 128C53; (B) A. tumefaciens C58(RP4). The lower, fainter band contains supercoiled plasmid DNA; the upper band contains nicked plasmid and linear chromosomal DNA.

plasmid DNA is observed. Second, the detection of plasmids in strains of Rhizobium using this technique is limited by the lysis procedure used.

Electron Microscopy of Plasmid DNA. Plasmid DNA from CsCl·EtBr density equilibrium gradients was examined by electron microscopy. An open-circular (OC) molecule of RP4 from A. tumefaciens strain C58(RP4) is shown in Figure 8. The molecular weight was calculated to be 35 ± 2 Mdal (mean ± S.D.) based on the contour length measurements of 14 molecules. This value agreed with the reported molecular weight of RP4 (41,52). Plasmid DNA molecules from CsCl·EtBr gradients of R. leguminosarum strain 128C53 plasmid DNA preparations were found to have a molecular weight of 119 ± 8 Mdal (Figure 9). This corresponds to the fastest migrating (smallest) plasmid of R. leguminosarum strain 128C53 (Figure 6), which migrated about the same distance into the gel as the 125-130 Mdal plasmid, pTiC58. The large size of the other two R. leguminosarum strain 128C53 plasmids and of the two plasmids of R. trifolii strain T37 would increase the probability of shearing of the plasmid DNA during preparation of the samples for electron microscopy. Thus, circular molecules of these plasmids were not observed when these samples were examined in the electron microscope.

Electron microscopy, like agarose gel electrophoresis can be used to determine both the size and the number of plasmids present in a strain. However, plasmids with molecular weight greater than 119 x 10^6 were not observed, probably due to shearing of the plasmid DNA molecules during preparation of the samples.

Plasmid Curing Experiments. Concurrent with the development of plasmid isolation techniques, experiments were performed with the

Figure 8. Electron micrograph of an open circular (OC) molecule of RP4. Plasmid DNA from A. tumefaciens strain C58(RP4) was isolated on a CsCl·EtBr density equilibrium gradient. The molecular weight was calculated to be 35 ± 2 Mdal based on an average contour length measurement of 16.9 ± 1 μM (14 molecules). Magnification: 2.64 x 10^4.

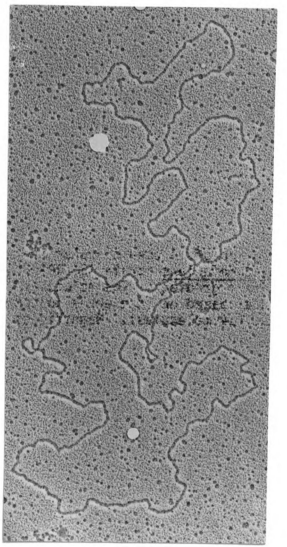
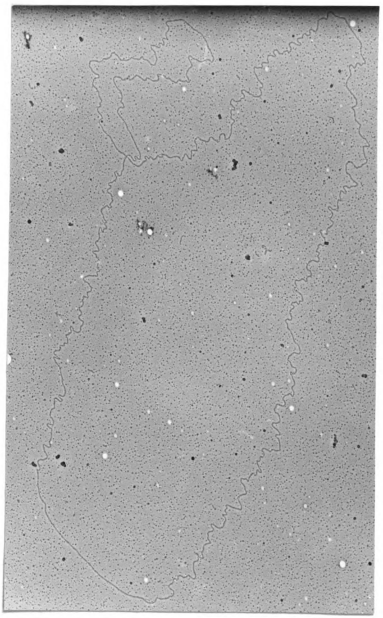


Figure 9. Electron micrograph of an open circular plasmid molecule from R. leguminosarum strain 128C53. Plasmids were purified by CsCl·EtBr density equilibrium centrifugation. The molecular weight was calculated to be 119 ± 8 Mdal from an average contour length measurement of 57.7 ± 3.3 μm (8 molecules). Magnification: 1.28 x 10^4.



intent of eliminating plasmids from strains of Rhizobium. The symbiotic properties (Nod^+ Fix^+) of isolates obtained from these experiments were then examined in order to correlate the loss of these properties with the absence of a specific plasmid.

Elimination of plasmids from R. "cowpea" strain 32H1 was attempted by growth at elevated temperature. The use of this technique has resulted in the loss of plasmids from Staphylococcus (124), Proteus (191), and several strains of Rhizobium (146,219). R. "cowpea" strain 32H1 was cultured for two days at 30°C and then shifted to an elevated temperature (37°C) for another 12 days of incubation. The viable cell density decreased only slightly over the period of growth at 37°C, although the turbidity (Klett units) increased for 4-5 days before leveling off (Figure 10). At the end of the treatment period, an aliquot was diluted and plated on YEM agar, and 50 colonies were picked.

The ability to induce dinitrogenase activity in cultures of free-living Rhizobium has been reported (110,193,144). This property was examined in wild-type R. "cowpea" strain 32H1 and in isolates grown for 12 days at 37°C. Wild-type strain 32H1 showed substantial activity after several days growth on CS7 medium (Figure 11). A loss of dinitrogenase activity was observed in 4% of the 50 isolates obtained from the culture grown at 37°C. All 50 isolates of strain 32H1 obtained prior to the temperature shift still retained dinitrogenase activity. Since the existence of plasmids in R. "cowpea" strain 32H1 could not be demonstrated, the loss of dinitrogenase activity in 4% of the "heat-treated" isolates could not be correlated with the loss of a specific plasmid. The loss of dinitrogenase activity was probably due to a mutation and not to the elimination of a plasmid.

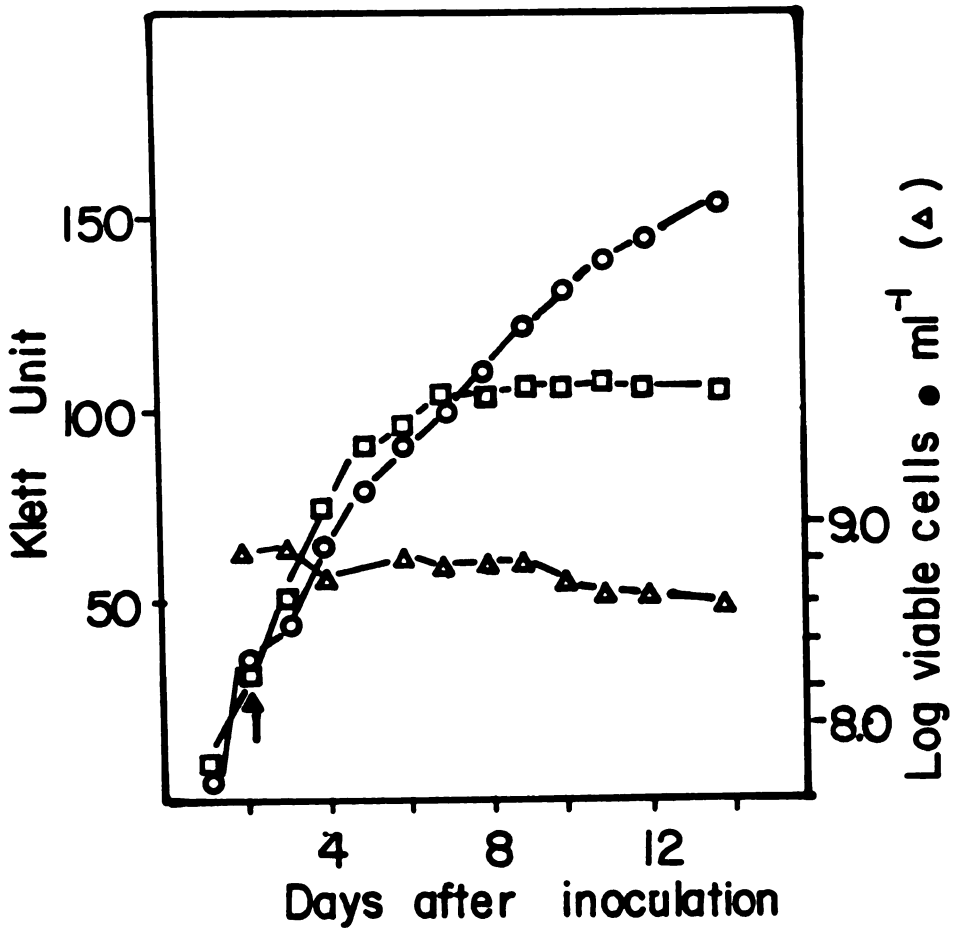


Figure 10. Growth of R. "cowpea" strain 32H1 at elevated temperature. Two 100-ml YEM cultures were inoculated with approximately 10^8 cells of R. "cowpea" strain 32H1. The cultures were grown at 30°C with shaking until the cell density was about 2 x 10^8 cells/ml (30 Klett units). On day 2, one flask (□) was shifted to 37°C (↑). The flasks were incubated another 12 days at 30°C (○) or 37°C (□). Viablity of cells grown at 37°C (Δ) was determined by plating suitable 10-fold serial dilutions on YEM agar.

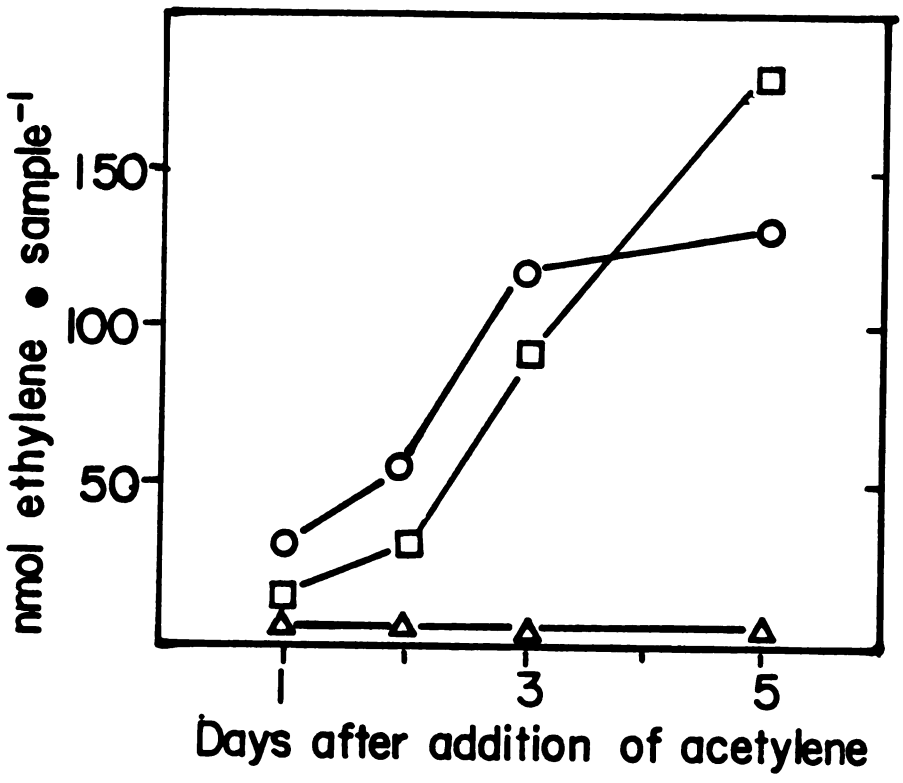


Figure 11. Acetylene reduction assays of free-living Rhizobium grown on CS7 medium. R. "cowpea" strain 32H1, (O); R. "cowpea" strain CB756, (□); and R. trifolii strain T37, (Δ).

Acridine dyes are effective in eliminating the F sex factor from Escherichia coli (88), and extrachromosomal DNA from yeast (183) and eukaryotic cells (21,186). The elimination is apparently due to interference by the dye in the replication of the plasmid (91,209). Nonnodulating mutants of Rhizobium have been isolated following treatment with acridine dyes (60,85,216). The Nod^- phenotype was not demonstrated to be due to the elimination of a plasmid, however.

Three strains of Rhizobium reportedly capable of nitrogen fixation in a free-living state were treated with the intercalating dye acridine orange. Cultures were grown for 24 h in the presence of various concentrations of acridine orange (Figure 12). Increasing concentrations of acridine orange resulted in a decrease in the number of viable cells present after 24 h of growth. In 20 µg/ml acridine orange, the cell concentration decreased below the concentration after inoculation for R. "cowpea" strain 32H1 and R. trifolii strain T37, indicating cell death. Isolates from cultures treated with acridine orange were tested for the loss of symbiotic properties. R. "cowpea" strain 32H1 and strain CB756 both reduced acetylene in culture on CS7 medium (Figure 11). Treatment of R. "cowpea" strain CB756 with 10 and 20 µg/ml acridine orange resulted in a frequency of loss of dinitrogenase activity similar to that observed for isolates not treated (Table 3). In contrast, 10 and 16% of the isolates of strain 32H1 treated with 10 and 20 µg/ml acridine orange, respectively, had lost the ability to reduce acetylene (Fix^-) in culture. The Fix^- isolates of strain 32H1 treated with acridine orange appeared larger and more mucoid than the Fix^+ colonies.

Figure 12. Treatment of strains of Rhizobium with various concentrations of acridine orange. Cell densities (cells/ml) in flasks containing acridine orange at the start of the 24 h incubation were: 8 x 10^3, R. "cowpea" strain 32H1, (O); 4 x 10^2, R. "cowpea" strain CB756, ([]); and 5 x 10^2, R. trifolii strain T37, (Δ).

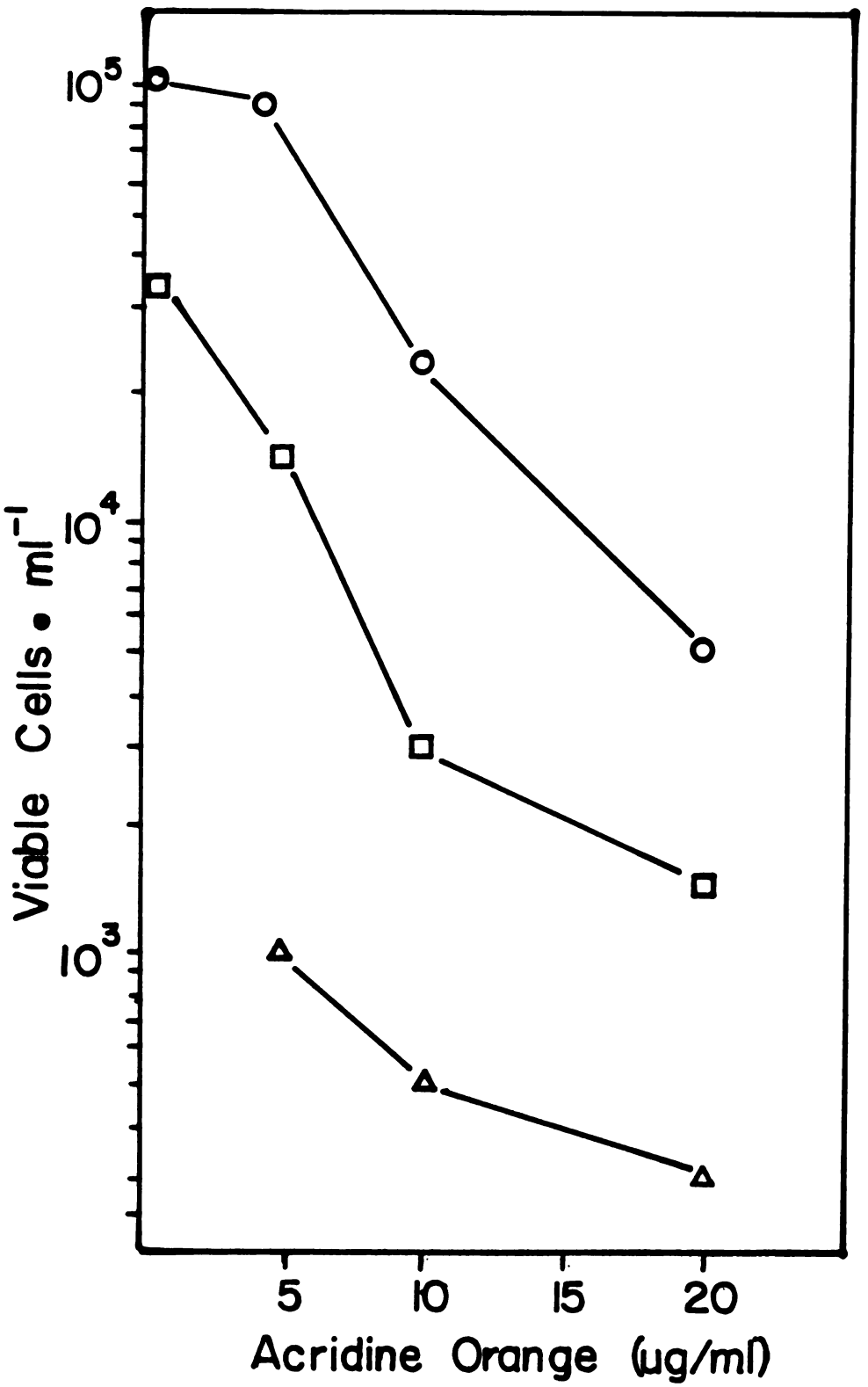

10^5
10^4
10^3
Viable Cells • ml^{-1}
5
10
15
20
Acridine Orange (µg/ml)

Table 3. *In vitro* nitrogen fixation by *Rhizobium* "cowpea" strains grown in the presence of acridine orange.

Strain	Acridine orange µg/ml	Number of colonies tested	% Fix⁻
32H1	0	185	3
	10	88	10
	20	150	16
CB756	0	91	2
	10	158	4
	20	99	2

Data were pooled from three experiments. Acetylene reduction assays were carried out as described in Methods.

R. "cowpea" strain 32H1 isolates were also examined for the ability to nodulate cowpea. Isolates (5 Fix^+, 5 Fix^-) from both untreated cultures and those containing 20 µg/ml acridine orange were used to inoculate cowpea plants. All untreated Fix^+ isolates formed effective nodules on cowpeas, as did 4 of the 5 Fix^+ isolates from the culture treated with 20 µg/ml acridine orange. All five acridine orange-treated Fix^- isolates and four of the five untreated Fix^- isolates were unable to nodulate cowpeas.

R. "cowpea" strain 32H1 is apparently more susceptible to acridine orange than strain CB756, since a higher frequency of isolates with mutant phenotypes were obtained upon treatment of strain 32H1 with acridine orange as compared with acridine orange treated isolates of strain CB756.

The Fix^- isolates of strain 32H1 were generally also Nod^-, suggesting that genes coding for nodulation and nitrogen fixation are linked in this strain. Elimination of a plasmid from these isolates of strain 32H1 could not be demonstrated. Therefore, the mutant phenotype may result from a deletion of either plasmid or chromosomal DNA, and may not be due to the elimination of a plasmid.

R. trifolii strain T37 cultures were also assayed for dinitrogenase activity, however, no activity was detected in cultures grown on CS7 medium (Figure 11). Cultures grown on a modified medium in which malic acid and glutamate were substituted for arabinose, succinic acid and glutamine (193) also lacked dinitrogenase activity. Reduction of the oxygen concentration in the vials to 1% failed to elicit dinitrogenase activity. Therefore, in lieu of assaying dinitrogenase activity in culture, the ability of acridine orange-treated isolates of R.

trifolii strain T37 to nodulate clover was determined. All untreated isolates nodulated clover plants within two weeks (Table 4). Half of the isolates treated with 20 μg/ml acridine orange and 35% of the isolates treated with 5 μg/ml did not nodulate clover plants two weeks after inoculation. After four weeks, however, all of the plants inoculated with acridine orange-treated isolates had formed effective nodules. Uninoculated plants did not have nodules; thus, the delayed nodulation was not due to contamination. Isolates exhibiting delayed nodulation and those nodulating normally were examined for plasmid DNA (Figure 13). No differences in the plasmid profiles were observed. Thus, the delay in nodulation was probably caused by an acridine orange-induced mutation and not as a result of the elimination of a plasmid. Whether this mutation was located on a plasmid or on the chromosome could not be determined.

Table 4. Nodulation of white clover by R. trifolii strain T37 grown in the presence of acridine orange.

Acridine orange condentration µg/ml	Number of colonies tested	% colonies forming nodules at:	
		2 weeks	4 weeks
0	24	100	100
5	20	65	100
20	4	50	100

Plants were grown in enclosed tubes on Jensen agar slants as described in Materials and Methods. Uninoculated plants were not nodulated after 4 weeks. Nodules on all plants reduced acetylene.

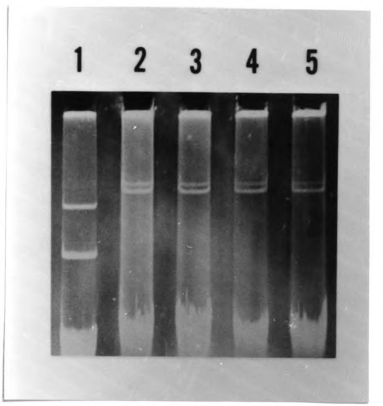


Figure 13. Agarose gel electrophoresis of plasmid DNA isolated using the alkaline lysis procedure from R. trifolii strain T37 isolates treated with acridine orange. Lane 1, A. tumefaciens strain C58(RP4); lanes 2 and 3, R. trifolii strain T37 isolates exhibiting normal nodulation; lanes 4 and 5, strain T37 isolates exhibiting delayed nodulation.

CHAPTER II

The Identification of a *Rhizobium trifolii* strain T37 Plasmid Coding for Nitrogen Fixation and Nodulation Genes and its Interaction with pJB5JI, a *Rhizobium leguminosarum* plasmid.

INTRODUCTION

The genetic and biochemical events involved in the establishment and maintenance of the Rhizobium - legume symbiosis are not well understood. Numerous reports, however, have indicated that many species of Rhizobium contain large plasmids, with molecular size > 100 megadaltons (Mdal), which encode symbiotic functions. Nitrogenase (nif) structural genes have been located on large plasmids in R. leguminosarum (137, 146), R. meliloti (5,164), and R. phaseoli (14). Genes involved in nodulation ability (nod) have also been found to be plasmid encoded (95,100,179,215), and are located on the same plasmid as the nif genes in R. meliloti (5,164), R. leguminosarum (94,147) and R. phaseoli (94).

Johnston et al. (100) identified a plasmid coding for pea nodulation in R. leguminosarum. This bacteriocinogenic plasmid transferred at high frequency (10^{-2}) into other Rhizobium and conferred the ability to nodulate peas to a Nod^- strain of R. leguminosarum and to heterologous species of Rhizobium. Similar results have been obtained by Brewin et al. (23) who reported that a non-bacteriocinogenic plasmid from a strain of R. leguminosarum transferred at low frequency (10^{-6}) and conferred pea nodulation ability.

Transfer of clover nodulation ability from a Nod^+Fix^+ R. trifolii strain to Nod^- strains has been achieved by transfer of a cointegrate plasmid composed of a broad-host-range plasmid and the R. trifolii nodulation plasmid (179). Transfer of a Tn 5-containing

derivative of a R. trifolii nodulation plasmid to other species of Rhizobium and to Agrobacterium tumefaciens has also been reported (95). Incompatibility between two Rhizobium plasmids has been used to identify a plasmid coding for symbiotic functions in R. phaseoli (14). Loss of the ability of R. phaseoli to nodulate beans correlated with the absence of a 190 Mdal plasmid in isolates into which a R. leguminosarum plasmid had been transferred.

Results presented in this chapter identify pRtT37a as the plasmid in R. trifolii strain T37 which codes for the nif genes. The loss of pRtT37a or formation of "hybrid" plasmids of various molecular weights from pR+T37a and pJB5JI, a R. leguminosarum plasmid coding for pea nodulation, was observed upon transfer of pJB5JI to R. trifolii strain T37. Symbiotic properties and plasmid profiles of strains generated upon transfer of R. leguminosarum plasmid pJB5JI to R. trifolii strain T37 were examined.

MATERIALS AND METHODS

Materials. Tryptone, yeast extract and Bacto agar were purchased from Difco Laboratories, Detroit, MI. Rifampicin, kanamycin sulfate, streptomycin sulfate, lysozyme, RNase (Type 1A), DNase I, polyethylene glycol (6000), pronase (Type XIV), ethidium bromide, Ficoll (400,000), bovine serum albumin (BSA, Fraction V), and all vitamins were obtained from Sigma Chemical Company, St. Louis, MO. Proteinase K was purchased from Calbiochem, La Jolla, CA. DNA polymerase I, and restriction endonucleases Hind III, Bam Hl and Bgl II were purchased from Bethesda Research Laboratories, Gaithersburg, MD. Xho I was purchased from P. L. Biochemicals, Milwaukee, WI. Eco Rl was a generous gift from Dr. A. Revzin, Michigan State University, East Lansing, MI. Polyvinylpyrolidone was obtained from GAF Corporation, New York, NY. [α-^{32}P]Labeled deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and thymidine triphosphate were purchased from Amersham Corporation, Arlington Heights, IL (800 Ci/mmole) or from New England Nuclear, Boston, MA (600 Ci/mmole). Agarose for routine use was obtained from Sigma (Type VI) or Bethesda Research Laboratories (gel electrophoresis grade). Agarose (low-m_r) used for the analysis of plasmid profiles by the Ekhardt agarose gel technique was purchased from BioRad Laboratories, Richmond, CA. Low melting temperature agarose (Sea plaque) was purchased from FMC Corporation, Rockland, ME. All other chemicals were reagent grade or were obtained

from sources described in Chapter I. Nitrocellulose (BA85, 0.45 μm; BA83, 0.2 μm) and a microfilter assembly were purchased from Schleicher and Schuell, Keene, NH. GF/C glass fiber filters (24 mm) and 3 MM filter paper were obtained from Whatman, Clinton, NJ. Filters (HAWP; 0.45 μm) to be used as a support for bacterial matings were purchased from Millipore Corporation, Bedford, MA. X-Ray film was purchased from Kodak, Rochester, NY. Dupont Cronex Lightning Plus intensifying screens were purchased from Picker Corporation, Detroit, MI.

Bacterial Strains, Plasmids, and Phage. The bacterial strains and plasmids used in this work are listed in Table 5 and 6, respectively. The nomenclature employed for designation of naturally occurring *Rhizobium* plasmids conformed to the format recommended by Novick *et al.* (136). *R. trifolii* plasmids are designated by pRt followed by the strain number and serial letters in cases of multiple plasmids: pRtT37a, pRt0403c.

Bacteriophage λ::Tn*5* was obtained from B. Chelm, Michigan State University, East Lansing, MI.

Media. YEM and YE2 media were described in Chapter I. TY medium (10) contained: 5.0 g tryptone, 3.0 g yeast extract; and 0.8 g $CaCl_2$ per liter redistilled water. The minimal medium for growth of *Rhizobium* was RM medium (119). RM medium consisted of : 10.0 g mannitol, 0.22 g K_2HPO_4, and 1.1 g sodium glutamate per liter redistilled water (pH 6.8). After autoclaving, 1.0 ml each of the vitamins and minor salts stock solutions was added per liter of medium. The vitamin stock solution consisted of a filter-sterilized solution of 10 mg thiamine-HCl and 20 mg biotin in 100 ml of 50 mM potassium phosphate, pH 7.0. The minor salts stock solution contained: 0.04 g

Table 5. Bacterial strains.

Bacteria	Relevant Properties	Source
Rhizobium trifolii		
T37	w.t., Nod^+Fix^+ on clover	(a)
0403	w.t., Nod^+Fix^+ on clover	(a)
6001-6140	T37 (pJB5JI) transconjugants; (kan^r)	(b)
7001-7051	0403 (pJB5JI) transconjugants; (kan^r)	(b)
Rhizobium leguminosarum		
T83K3	*phe trp* rif^r str^r Nod^+Fix^+ on pea contains pJB5JI (kan^r)	(c)
726	rif^r Nod^+Fix^+ on pea	(c)
Agrobacterium tumefaciens		
C58(RP4)	contains RP4, pTiC58, pAtC58	(d)
Pseudomonas aeruginosa		
PAO2	contains pMG1	(e)
PAO2	contains pMG5	(e)
Escherichia coli		
ED8654	*met gal* hsd_K R^- M^+ *supE supF* λ::Tn*5* host	(f)
AB2880	contains pSA30 (tet^r)	(g)
HB101	contains pRmR2 (tet^r)	(g)

(a)F.B. Dazzo, Michigan State University, East Lansing, MI
(b)This work
(c)A.W.B. Johnston, John Innes Institute, Norwich, England
(d)T.C. Currier, Kansas State University, Manhattan, KS
(e)R.H. Olsen, University of Michigan, Ann Arbor, MI
(f)B. Chelm, Michigan State University, East Lansing, MI
(g)F.M. Ausubel, Massachusetts General Hospital, Boston, MA

Table 6. Plasmids

Plasmids	Relevant Properties	Reference
pJB5JI	encodes *R. leguminosarum nif* genes, pea *nod* genes; contains Tn *5* (kan^r); 130 Mdal	(100)
pSA30	*Klebsiella pneumoniae nif* genes; 10 kb	(34)
pRmR2	*R. meliloti nif* genes; 8 kb	(166)
RP4	kan^r tet^r amp^r; 35 Mdal	(41)
pTiC58	tumor-inducing plasmid; 130 Mdal	(41)
pAtC58	325 Mdal	(35)
pMG1	312 Mdal	(75)
pMG5	280 Mdal	(75)

$CaCl_2$, 0.033 g $FeCl_3 \cdot 6H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, and 0.83 ml 12 N HCl in 99 ml redistilled water.

E. coli and Pseudomonas aeruginosa strains were grown at 37°C on LB medium (130) composed of: 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter redistilled water. For the propagation of λ::Tn5, LB medium was supplemented with 1 M $MgSO_4 \cdot 7H_2O$ to a final concentration of 10 mM.

Media were solidified with 1.5% Bacto agar. Soft LB agar consisted of LB medium plus 0.7% Bacto agar.

Plasmid Transfer. Bacterial matings were carried out essentially as described by Beringer et al. (13). Bacteria were grown for three days on TY medium agar slants. The bacteria were washed from the slants with 2.5 ml sterile 10 mM $MgSO_4$ and matings were performed by mixing 0.5 ml (about 5×10^8 cells) of donor and recipient cells. The mixed cell suspensions were pipetted onto 0.45 μm Millipore filters on non-selective TY medium agar plates. The plates were incubated for 24 h at 30°C. Filters were transferred to 3-dram vials and cells were resuspended in 2.5 ml sterile 10 mM $MgSO_4$. Appropriate 10-fold serial dilutions in 10 mM $MgSO_4$ were plated on selective medium. Transfer frequencies were calculated per recipient cell plated. Transconjugant colonies were restreaked on selective medium to obtain single colony isolates.

Ekhardt Agarose Gel Electrophoresis Technique. Plasmid profiles of donor, recipient and transconjugant strains were obtained using a modification of the Ekhardt agarose gel electrophoresis technique (63). Bacteria were grown without shaking in 5 ml YE_2 medium for 48 h at 30°C. Cells (10^7 - 10^8) were centrifuged, washed with 0.1% sarkosyl

in TE buffer and resuspended in 20 µl of modified Ekhardt gram-negative bacteria lysis buffer. The buffer contained 2.0 mg lysozyme, 5.0 mg bromphenol blue, 2.0 mg Ficoll (400,000) and a 10-fold greater amount of RNase (0.5 mg) in 10 ml of TBE electrophoresis buffer. Immediately after resuspension, cells were carefully layered beneath 150 µl of Ekhardt SDS solution (0.2% SDS, 10% Ficoll in TBE buffer) in a well (3 mm x 6 mm) of a 3 mm thick vertical 0.7% agarose gel. Plasmid DNA was separated by electrophoresis for 1 h at 8 mA followed by 10 - 12 h at 20 mA. Alternatively plasmid DNA was rapidly separated by electrophoresis for 1 h at 8 mA followed by 3 h at 40 mA. The sizes of the plasmids were determined from their relative mobilities in agarose gels (129) using RP4 (35 Mdal), pTiC58 (130 Mdal), pJB5JI (130 Mdal), pMG5 (280 Mdal), pMG1 (312 Mdal), and pAtC58 (325 Mdal) as size markers.

Nodulation Tests. The symbiotic properties of strains of Rhizobium were tested on white clover (Trifolium repens var. Ladino) and pea (Pisum sativum var. Wisconsin Perfection). Clover nodulation tests were carried out in enclosed tubes as described in Chapter 1. Pea nodulation tests were carried out on partially enclosed plants (201). Pea seeds were surface sterilized as described for clover seeds and germinated on water agar plates. Pea seedlings were transferred to sterile, cotton-plugged, 25 x 200 mm culture tubes containing Vermiculite wetted with 30 ml of Fahraeus medium containing: 0.2 g $CaCl_2$, 0.22 g $MgSO_4 \cdot 7H_2O$, 0.1 g KH_2PO_4, 0.15 g Na_2HPO_4, 0.005 g iron citrate and 1 ml BMM medium trace element stock solution (see Chapter 3) per liter redistilled water (pH 6.5). Pea seedlings were inoculated as described for clover seedlings and the plants were grown under the same conditions. After approximately one week, the shoot of

the pea plant was gently pulled past the cotton plug. Additional sterile Fahraeus medium was added three weeks after inoculation. After five weeks, plants were scored for root nodules and were tested for acetylene reduction ability.

Acetylene Reduction Assay. Dinitrogenase activity was assayed by measuring the amount of ethylene produced from the reduction of acetylene (78). Acetylene reduction assays on pea plants were carried out essentially as described for clover and cowpea plants (Chapter 1). The shoot of the pea plant was excised and the culture tubes were sealed with serum caps. Air (5.0 ml) was removed and replaced with an equal volume of acetylene and samples (0.2 ml) were taken after 15 min incubation at room temperature. Samples were analyzed for ethylene by gas chromatography as described in Chapter I.

Isolation of Bacteria from Root Nodules. Rhizobium were isolated from clover and pea root nodules essentially as described by Vincent (201). Nodules were clipped from the plant with a small portion of the root still attached. The detached nodules were placed in sterile 3-dram vials and washed several times with 10 mM $MgSO_4$. The nodules were surface sterilized by covering with 95% ethanol for 30 seconds, and then treating with 5% (v/v) hydrogen peroxide (300 µl) for 2 min. The hydrogen peroxide solution was replaced with 150 µl sterile 10 mM $MgSO_4$ and the nodules were thoroughly crushed with the flattened end of a sterile glass rod. The nodule suspension was spread onto the surface of YEM agar plates (containing antibiotic, if desired) and then incubated at 30°C for 4-5 days. Bacteria were restreaked to obtain single colony isolates.

Fahraeus Slide Technique. The infection of clover root hairs by R. trifolii bacteria was studied using a simple glass slide procedure (65). Bacteria were grown for 3 days on TY agar plates. Cells were aseptically washed from the plates with 1 M NaCl, 10 mM EDTA and pelleted by centrifugation. The bacteria were washed with sterile Fahraeus medium and resuspended to a density of about 10 Klett units (10^8 cells/ml) in sterile Fahraeus medium. An aliquot (0.4 ml) of the cell suspension was pipetted onto one end of a sterile microscope slide (25 mm x 75 mm). Two 1-day-old clover seedlings were placed on the microscope slide with roots (1 cm in length) projecting into the cell suspension. A sterile cover slip (25 mm x 40 mm) was carefully placed over the clover roots leaving the clover hypocotyl uncovered. The microscope slide was placed in a sterile covered tube containing 7 ml of Fahraeus medium. The tubes were incubated in a growth chamber as previously described for clover plants. The clover roots were examined with a phase contrast microscope at 24 h intervals.

Isolation of Total DNA. Starter cultures of bacteria were grown in 10 ml YE_2 medium for 3 days. An aliquot (2 ml) was used to inoculate 200-ml cultures of YE_2 medium. After growth for 24 h, the cells were pelleted by centrifugation, washed with 0.1% (w/v) Sarkosyl in TE buffer, and resuspended in 2.5 ml STE buffer. Lysis was carried out by the addition of 2 ml "sucrose mix" solution (1.6 M sucrose, 0.55 M Tris, pH 8.0, 0.1 M EDTA), 1 ml 5 mg/ml lysozyme, and 22 ml 10 mM EDTA (177). The lysate was incubated on ice for 20 min at which time 15 ml 2.5% (w/v) Sarkosyl was added. The lysate was incubated for 20 min on ice, extracted twice with phenol, reextracted twice with chloroform, and digested for 2 h at 37°C with 20 µg/ml RNase (1 mg/ml in 0.4 M

sodium acetate, pH 4.0; boiled 10 min). The RNase-digested lysate was reextracted with phenol and chloroform. The aqueous phase was recovered and the DNA was ethanol precipitated. The DNA was pelleted by centrifugation and resuspended in TE buffer. The DNA concentration was determined by the diphenylamine assay.

Determination of DNA Concentration. The concentration of DNA was determined by absorbance at 260 nm or by the diphenylamine method (70). DNA samples (500 µl) containing 10% (v/v) perchloric acid were mixed with an equal volume of 4% (w/v) diphenylamine in glacial acetic acid. An aliquot (25 µl) of aqueous acetaldehyde (1.6 mg/ml) was added and the samples were incubated for 10 - 12 h at 37°C. The DNA concentrations in the samples were determined by measuring the absorbance at 595 nm. The assay was linear between 0 - 20 µg calf thymus DNA. The concentrations of small amounts of DNA (<250 ng/ml) were estimated by the comparison of the intensity of fluorescence of the EtBr-stained unknown DNA with that of EtBr-stained DNA standards in agarose gels (121). The unknown DNA sample and a series of standard DNA solutions were electrophoresed on an agarose minigel. The gel was stained with ethidium bromide and photographed. The quantity of DNA in the unknown sample was estimated by eye from the gel photo.

Agarose Gel Electrophoresis. DNA samples were separated electrophoretically on vertical agarose gels as described in Chapter I. Horizontal agarose gels (12.5 cm x 18 cm x 1 cm) were electrophoresed at 60 volts for 15 h. Small amounts of DNA (20 - 50 ng) could be analyzed rapidly by electrophoresis on a mini agarose gel apparatus (121). Electrophoresis of DNA samples on a mini agarose gel (8.5 cm x

6.5 cm x 0.3 cm) was carried out at 100 volts for 45-60 min. Gels were stained and photographed as described previously (Chapter I).

Growth of Bacteriophage λ::Tn 5. E. coli strain ED8654 was used as a host for bacteriophage λ::Tn 5. Overnight cultures of strain ED8654 grown on LB medium were centrifuged at 3000 x g for 10 min at 4°C. The pellet of bacteria was resuspended in 0.4 volume sterile 10 mM $MgSO_4$. This cell suspension was used to inoculate broth cultures and to determine bacteriophage titers. Titers were determined by mixing 0.1 ml of ten-fold serial dilutions of phage solution with 0.1 ml of the cell suspension. The bacteria/phage mixture was incubated at 37°C for 20 min, then combined with 3 ml soft LB agar at 45°C and plated on LB agar plates. After 12 h at 37°C, the plates were scored for plaques.

A plate lysate stock solution of bacteriophage λ::Tn 5 was prepared by plating approximately 10^5 plaque-forming units (pfu) with 0.1 ml of the bacterial cell suspension, as described above. Phage from the confluent lysis were recovered by adding 10 ml SM phage buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 0.05% gelatin) to the plate and storing at 4°C for 12 h. The SM buffer containing the phage was decanted from the plate and 0.1 ml chloroform was added. After brief vortexing, the SM buffer was centrifuged at 4000 x g for 10 min at 4°C. The supernatant fluid was recovered, and chloroform was added to a final concentration of 0.3%. The titer of the plate lysate stock solution of λ::Tn5 was determined to be 5 x 10^9 pfu/ml.

Large scale growth of phage λ::Tn5 was carried out as described (18,210). An overnight culture (about 10^9 cells/ml) of strain ED8654 in 10 ml LB medium was supplemented with 1 M $MgSO_4$ to a final

concentration of 10 mM $MgSO_4$. Phage plate lysate stock solution (0.2 ml) was added and the mixture was incubated at 37°C for 20 min. The phage/bacteria mixture was transferred to a 500-ml culture of LB medium + 10 mM $MgSO_4$ equilibrated to 37°C. The culture was incubated at 37°C with shaking for 10 h, at which time lysis was evident. Chloroform (5 ml) was added to the lysate and the flask was shaken 15 min. Cellular debris was removed by centrifugation at 3000 x g for 30 min at 4°C. The supernatant fluid (titer = $8x10^{10}$ pfu/ml) was decanted into a 1-liter flask and digested with DNase (1 µg/ml) and RNase (1 µg/ml) for 1 h at 4°C. PEG 6000 (50 g) and NaCl (29.22 g) were added and the solution was stirred for 15 min. The solution was allowed to stand for 2 h and then centrifuged at 3000 x g for 40 min at 4°C. The phage pellet was resuspended in 2.5 ml TM buffer and was mixed until well dispersed. The phage suspension was extracted with an equal volume of chloroform to remove the PEG, and the aqueous phase containing the phage was recovered. The phage solution was layered on a 5% to 40% glycerol step gradient (197) and centrifuged at 35,000 rpm for 60 min at 4°C in a Beckman SW41 rotor. The pellet of phage particles was resuspended in 1 ml TM buffer. The phage was further purified on a CsCl step gradient (ρ = 1.7 g/ml, 1.5 g/ml, 1.45 g/ml). The gradient was centrifuged in a Beckman SW41 rotor at 37,000 rpm for 2 h at 4°C. The gradient was fractionated and the absorbance at 260 nm was used to determine which fractions contained phage.

Isolation of DNA from Phage Particles. Cesium chloride gradient fractions containing phage were pooled and dialyzed for 2 h at room temperature against 0.1 M Tris (pH 8.0), 0.15 M NaCl, 1 mM $MgCl_2$ (2 liter). DNA was extracted from the phage particles by digestion at

68°C for 1 h in a solution containing 50 μg/ml Proteinase K, 0.1% SDS, and 10 mM EDTA. The phage DNA solution was extracted twice with an equal volume of phenol/chloroform (1:1,v/v), twice with chloroform, and once with ether. The phage DNA was ethanol precipitated and resuspended in 1 ml TE buffer. From two 500-ml cultures, 720 μg of λ::Tn 5 DNA was obtained.

Cleared-Lysate Procedure for Plasmid Isolation. The small amplifiable plasmids, pSA30 and pRmR2, were isolated using the cleared-lysate technique (37). A 10-ml culture of LB medium containing the appropriate antibiotic was inoculated with the E. coli strain harboring either pSA30 or pRmR2. The culture was incubated overnight at 37°C with shaking and was used to inoculate a 1-liter culture of LB medium + antibiotic. The culture was grown at 37°C until the OD_{600} was between 0.4 and 0.6. Chloramphenicol (150 mg) was added to amplify the plasmid (36) and the incubation was continued for 15-18 h. Bacterial cells were harvested by centrifugation at 4000 x g for 10 min at 4°C. The cells were washed with 20 ml 25% (w/v) sucrose in TE buffer, and resuspended in 50 ml 25% sucrose in TE buffer. Lysozyme (7 ml of a 5 mg/ml solution in 0.25 M Tris, pH 8.0) was added and the solution was incubated on ice. After five min, 15 ml 0.25 M EDTA was added. The solution was mixed gently and incubated on ice for 5 min. Cells were lysed by the addition of 8 ml 10% (w/v) SDS and the solutions were mixed by gentle inversion. The cleared-lysate was adjusted to 1 M NaCl with 20 ml 5 M NaCl. The solution was incubated on ice for 90 min and was centrifuged at 30,000 x g for 30 min at 4°C. The supernatant fluid containing plasmid DNA was decanted, extracted with an equal volume of phenol (equilibrated with TE buffer) and reextracted with chloroform.

The aqueous phase was recovered and DNA was precipitated by the addition of 0.1 volume 3 M sodium acetate and 2 volumes 95% ethanol. The DNA was pelleted by centrifugation at 12,000 x g for 20 min at 4°C. The DNA pellet was resuspended in 1 ml TE buffer and was further purified by CsCl·EtBr density equilibrium centrifugation as described in Chapter 1.

Isolation of DNA Fragments from Agarose Gels. DNA fragments were isolated from agarose gels by electrophoresis onto dialysis membranes (71,121). An incision was made in the agarose gel directly in front of the desired DNA band. A piece of Whatman 3 MM filter paper and a piece of dialysis membrane were inserted into the incision with the filter paper between the dialysis membrane and the DNA band. Electrophoresis was carried out until the DNA had migrated into the 3 MM paper. The dialysis membrane and the 3 MM paper were inserted into a 500-µl microcentrifuge tube with a hole in the bottom. The tube was inserted into a 1.5-ml microcentrifuge tube and centrifuged for 15 sec to force the buffer containing the DNA fragment into the 1.5-ml tube. The filter paper and dialysis membrane were washed three times with 100 µl elution buffer (0.2 M NaCl, 50 mM Tris, pH 7.6, 1 mM EDTA, and 0.1% SDS). The eluates were pooled and extracted with equal volumes of phenol, phenol/chloroform (1:1, v/v) and chloroform. The DNA was ethanol precipitated and pelleted by centrifugation. The DNA was resuspended in 20 to 50 µl TE buffer and contaminating traces of agarose were removed by filtration of the DNA solution through a BA83 nitrocellulose filter using a microfilter apparatus.

Alternatively, DNA fragments were isolated from low-melting temperature agarose (Sea Plaque) gels (121,204). The segment of gel

containing the desired DNA fragment was cut out of the gel and was melted at 68°C in 5 volumes TE buffer. The melted agarose was extracted with equal volumes of phenol, phenol/chloroform, and chloroform. The DNA was recovered by ethanol precipitation and traces of agarose were removed as described above.

Preparation of nif Probe DNA. The 3.4 kb Eco RI-Hind III fragment of plasmid pSA30 containing the region of K. pneumoniae nif DNA homologous to the nif structural genes of other N_2-fixing bacteria or the 2.0 kb Eco RI-Xho I fragment of pRmR2 containing R. meliloti nif was used as a probe for Rhizobium nif DNA (Figure 14). Plasmid DNA was isolated from bacteria by the cleared-lysate technique and purified by ethidium bromide-cesium chloride centrifugation. Plasmid DNA was digested with restriction endonucleases according to manufacturer's specifications and the resulting fragments were separated by agarose gel electrophoresis. The desired fragments were recovered from the gel and 1 μg amounts were ^{32}P-labeled by nick translation.

Isolation of pRtT37a and pJB5JI. Crude extracts of plasmid were isolated from R. trifolii strain T37 and R. leguminosarum strain T83K3 using the alkaline lysis procedure (Chapter I). Plasmid DNA was separated from chromosomal DNA and other plasmids by electrophoresis on vertical 0.7% low-melting-temperature agarose gels. Segments of the gel containing pRtT37a and pJB5JI were excised and the DNA was recovered as described above. The DNA was ^{32}P-labeled by nick translation and used as a hybridization probe.

^{32}P-Labeling of DNA by Nick Translation. DNA was labeled in vitro with ^{32}P-labeled deoxynucleotide triphosphates (dNTPs) by the nick translation method (122,158). Labeled dNTPs (100 μCi) were

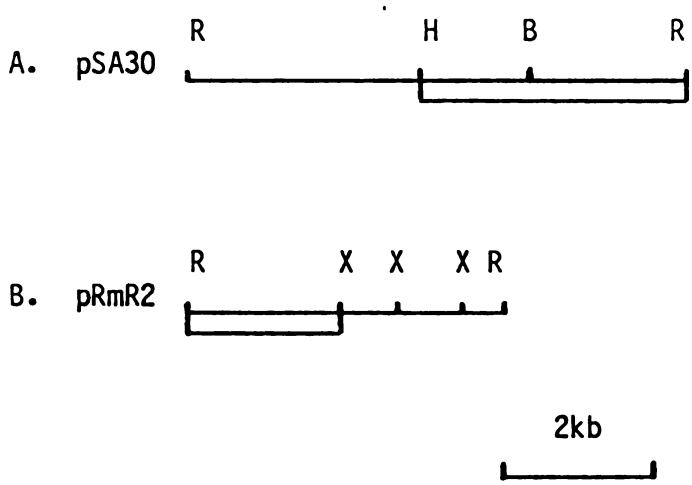


Figure 14. Partial restriction map of nif DNA cloned in pSA30 and pRmR2. A, endonuclease cleavage sites for Eco RI (R), Hind III (H), and Bam H1 (B) are given for K. pneumoniae nif DNA cloned in pSA30 (34). B, Eco RI (R) and Xho I (X) cleavage sites are indicated for R. meliloti nif DNA cloned in pRmR2 (166). The region of nif homology is indicated by the open bar.

pipetted into a 500-µl Eppendorf tube. DNA (1 µg) and 2.5 µl 10X nick translation buffer (0.5 M Tris, pH 7.5, 0.1 M $MgSO_4$, 10 mM DTT, 500 µg/ml BSA) were mixed with the dNTPs. The volume of the reaction mixture was adjusted to 23 µl with redistilled water. DNase I (1 µl of a 10^4-fold dilution in redistilled water of 1 mg/ml stock solution) and 1 µl of E. coli DNA polymerase (1 unit/µl) were added. The reaction was incubated for 2 h at 16°C. The reaction mixture was brought to room temperature and 2.5 µl 5 M NaCl, 2.5 µl 0.5 M EDTA, 1 µl 10% SDS, and 2.5 µl 5 mg/ml proteinase K in redistilled water were added. The reaction was incubated at 68°C for 30 min. Single stranded salmon sperm DNA (100 µl of 10 mg/ml redistilled water), yeast tRNA (100 µl of 1 mg/ml redistilled water) and 0.1 volume 3 M sodium acetate were added. The DNA was precipitated with the addition of 2 volumes of ethanol to separate the labeled DNA from the unincorporated nucleotides. Alternatively, unincorporated nucleotides were removed by the "spun column" procedure (121). A Sephadex G-50 column (0.9 ml bed volume) equilibrated with STE buffer was prepared in a disposable plastic syringe (1 ml). The nick translation reaction mixture was brought to 100 µl with STE buffer, and centrifuged through the column. The effluent was collected in an Eppendorf tube and contained the labeled DNA. Unincorporated nucleotides remained in the column. An aliquot (1 µl) of the labeled DNA solution was spotted on a GF/C filter and radioactivity was determined by liquid scintillation spectrometry. Specific activities of 5 to 6 x 10^7 dpm/µg DNA were routinely obtained.

Southern Hybridization. Transfer of DNA from agarose gels to nitrocellulose filters was carried out essentially as described by Southern (185). Prior to transfer, DNA was partially depurinated by

treatment with 300 ml 0.25 M HCl for 15 min (202), denatured for 30 min in 300 ml 0.3 M NaOH, 1.5 M NaCl, and neutralized for 60-90 min in 300 ml 0.5 M Tris (pH 7.0), 3 M NaCl. DNA transfer was carried out for 48 h using 6X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate). Filters were rinsed with 6X SSC, air dried and vacuum-baked for 2 h at 80°C. Filters were pretreated in sealed plastic pouches for at least 6 h at 68°C with hybridization buffer: 6X SSC, 0.5% SDS, 1 mM EDTA, 2X Denhardts solution (51), and 100 µg/ml denatured salmon sperm DNA. The pretreatment solution was removed and fresh hybridization buffer containing denatured ^{32}P-labeled probe DNA was added. The pouches were resealed and incubated at 68°C for 24 h. The filters were washed at 68°C in 300 ml 2X SSC, 0.5% (w/v) SDS (3 changes). The filters were rinsed a final time in 2X SSC at room temperature and then air dried. The filters washed under stringent conditions were treated with 300 ml 0.1X SSC, 0.5% SDS (300 ml) at 68°C (3 changes), rinsed with 300 ml 0.1X SSC and air dried. Autoradiography was carried out at -80°C for 3-5 days using Kodak X-Omat AR film with a Dupont Cronex Lightning Plus intensifying screen.

RESULTS

R. trifolii strain T37 and strain 0403 were analyzed for plasmid DNA using the Ekhardt agarose gel technique. At least three large plasmids were present in strain T37. Four large plasmids were detected in strain 0403.

The molecular weights of these plasmids were estimated from the relative mobilities of the plasmids in agarose gels. The relative mobilities of plasmids of known molecular weight were determined and a standard curve was constructed (Figure 15) (129). Two linear regions were observed in the curve: from 36×10^6 to 130×10^6 and from 280×10^6 to 325×10^6. The molecular weights of plasmids greater than 325×10^6 were estimated by extrapolation of the curve. The molecular weight of the smallest plasmid in *R. trifolii* strain T37, pRtT37a, was about 275×10^6, while pRtT37b and pRtT37c had molecular weights of about 300×10^6 and 380×10^6, respectively. The molecular weight of pRt0403a was about 290×10^6 and the molecular weights of pRt0403b, c, and d were estimated to be 335×10^6, 350×10^6, and 390×10^6, respectively.

Transfer Frequency of pJB5JI. The frequencies of transfer of Tn *5*-encoded kanamycin resistance located on pJB5JI from *R. leguminosarum* strain T83K3 to three strains of *Rhizobium* were determined (Table 7). Comparable frequencies of transfer of pJB5JI into *R. trifolii* strain T37 and strain 0403 were observed. Transfer of pJB5JI

Figure 15. Molecular weight of plasmid DNA versus log relative mobility of plasmids in agarose gels. x, RP4; *, pR1T83K3a; □, pR1128C53a; Δ, pJB5JI; O, pTiC58; ■, pMG5; ▲, pMG1; ●, pAtC58. Relative mobilities of plasmids of R. trifolii strain T37 and strain 0403 are indicated by the arrows. The linear portions of the curve, ——,; extrapolations of the curve, – – – –.

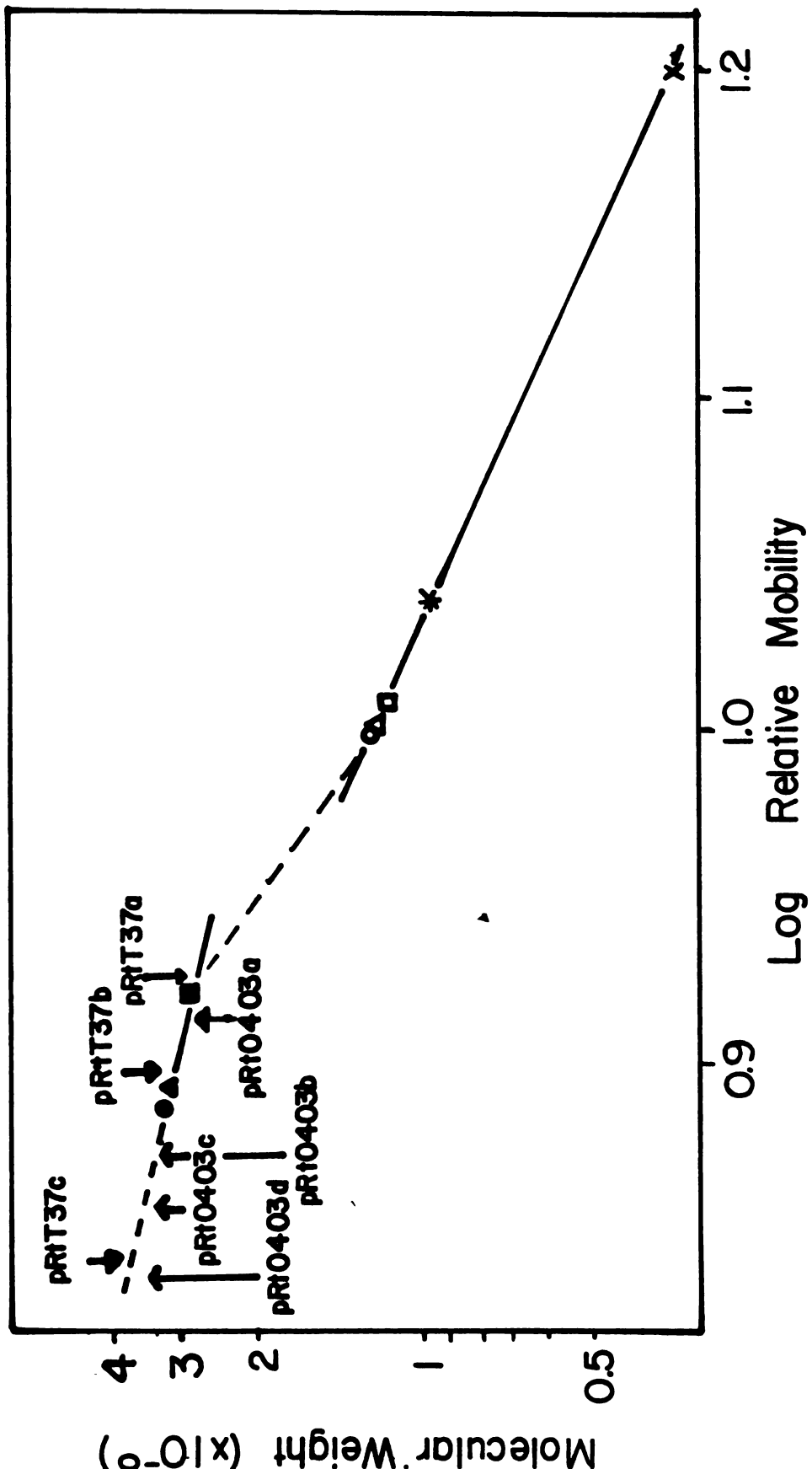

pRT37c
pRT37b
pRT37a
pRT0403c
pRT0403a
pRT0403d
pRT0403b
4
3
2
1
0.5
0.9
1.0
1.1
1.2
Log Relative Mobility
Molecular Weight (x10⁻⁸)

Table 7. Transfer of pJB5JI from *R. leguminosarum* strain T83K3

Recipient Strain	Frequency
R. trifolii strain T37	2.5×10^{-5}
R. trifolii strain 0403	2.4×10^{-5}
R. leguminosarum strain 726	5×10^{-3}

Transconjugants were selected on RM medium containing 200 μg/ml kanamycin. The transfer frequency was determined per recipient cell. The frequency of spontaneous resistance of all three recipient strains to 200 μg/ml kanamycin was $< 10^{-8}$. The frequency of reversion to auxotrophy of *R. leguminosarum* strain T83K3 was $< 10^{-8}$.

into R. leguminosarum strain 726 occurred at a frequency 200-fold greater than the frequencies observed for the R. trifolii strains. The frequency of transfer of pJB5JI into R. leguminosarum strain 726 was similar to frequencies of transfer of pJB5JI into other species of Rhizobium (100). R. leguminosarum strain T83K3 contains a second transmissible plasmid (100 Mdal) which transfers at a frequency of about 10^{-6} (101). This plasmid is one of two plasmids in the fastest migrating band of the donor strain (23) and was observed to cotransfer with pJB5JI into R. trifolii strain T37 and strain 0403 at a frequency of 7 to 8 x 10^{-6} per recipient cell.

Characterization of R. trifolii Strain 0403 (pJB5JI) Transconjugants. Fifty kanamycin-resistant colonies from crosses between R. leguminosarum strain T83K3 and R. trifolii strain 0403 were restreaked on selective medium and a single colony from each plate was selected. The strains (numbered consecutively from 7001-7050) were analyzed for plasmid DNA using the Ekhardt agarose gel electrophoresis technique.

The four plasmids indigenous to R. trifolii strain 0403 were present in all of the transconjugants and no alterations in the electrophoretic mobilities of these plasmids were observed (Figure 16). All of the transconjugants (except strain 7004) contained pJB5JI and 14 strains also contained the 100 Mdal plasmid (pR1T83K3a) of the donor strain. Strain 7004 was kanamycin-resistant yet lacked a plasmid band correspoding to pJB5JI. However, the intensity of fluorescence of the 100 Mdal plasmid in agarose gels of strain 7004 was increased, suggesting that approximately 30 Mdal of DNA had been deleted from pJB5JI (Figure 16, lane 4).

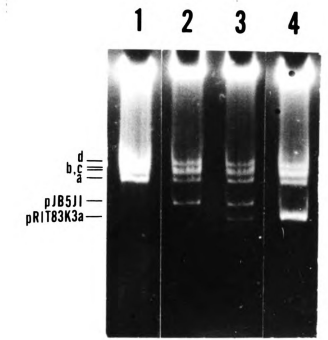


Figure 16. Ekhardt agarose gel electrophoresis of plasmid DNA of R. trifolii strain 0403 (pJB5JI) transconjugants. (1), R. trifolii strain 0403; (2), strain 7001, harboring pJB5JI; (3), strain 7014, harboring pJB5JI and pRlT83K3a; (4), strain 7004, harboring pRlT83K3a and plasmid-deletion mutant of pJB5JI.

The symbiotic properties of 14 of the transconjugants were examined on pea and clover plants. Thirteen of the transconjugants contained pJB5JI in addition to the four indigenous plasmids of R. trifolii strain 0403. Three of these strains also harbored pR1T83K3a. The other strain (7004) contained a derivative of pJB5JI in which about 30 Mdal of plasmid DNA was apparently deleted. Southern hybridization analysis using a heterologous nif probe demonstrated that the nif structural genes were also located on this region of deleted DNA (Figure 17, lane 4).

All of the transconjugants formed effective nodules on clover plants. Nodulation of the clover plants was not delayed when compared to R. trifolii strain 0403, and occurred within two weeks. The number of nodules produced on the plant roots were also similar.

All of the transconjugants (except strain 7004) formed nodules on pea plants. Fewer nodules were produced, however, and the nodules were small, white and unable to reduce acetylene (Fix^-). No nodules were produced on pea plants inoculated with strain 7004, suggesting that a gene(s) coding for pea nodulation ability were located on the 30 Mdal segment of pJB5JI DNA that was deleted.

Plasmid Analysis of R. trifolii Strain T37 (pJB5JI) Transconjugants. Initially, 40 kanamycin-resistant transconjugants from crosses between R. leguminosarum strain T83K3 and R. trifolii strain T37 were analyzed for plasmid DNA as described for the R. trifolii strain 0403 (pJB5JI) transconjugants. In contrast to the low number of different plasmid profiles obtained upon transfer of pJB5JI to strain 0403, a wide variety of plasmid profiles were observed for the strain T37 (pJB5JI) transconjugants (Figure 18). To further investigate the

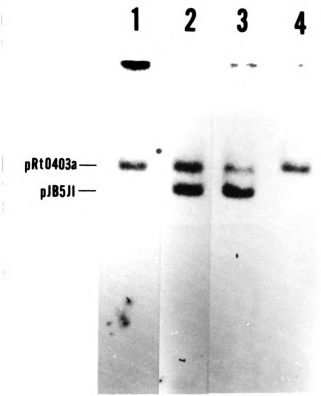


Figure 17. Autoradiogram of ^{32}P-labeled pRmR2 to a Southern filter of plasmid DNA from R. trifolii strain 0403 (pJB5JI) transconjugants. Plasmid DNA from an agarose gel similar to the one shown in Figure 15 was transferred to a nitrocellulose filter and hybridized to ^{32}P-labeled R. meliloti nif DNA. Lane 1, R. trifolii strain 0403; 2, strain 7001, harboring pJB5JI; 3, strain 7014, harboring pJB5JI and pR1T83K3a; 4, strain 7004, harboring pR1T83K3a and a deletion derivative of pJB5JI.

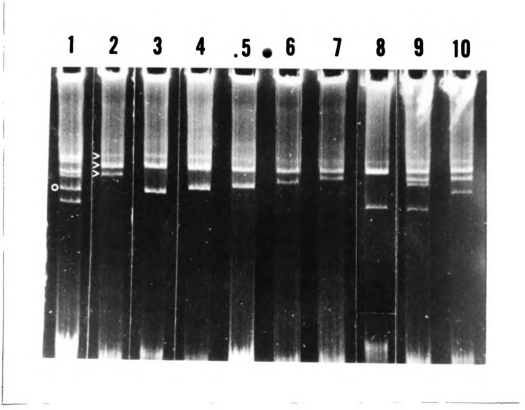


Figure 18. Ekhardt agarose gel electrophoresis of plasmid DNA of R. trifolii strain T37 (pJB5JI) transconjugants. (1), R. leguminosarum strain T83K3; (2), R. trifolii strain T37; (3-10), strain T37 (pJB5JI) transconjugant strains: 6032, 6060, 6012, 6076, 6067, 6130, 6135, 6137. The plasmid pJB5JI is noted with the symbol (0) and pRtT37a, pRtT37b, and pRtT37c are marked with the symbol (>).

occurrence of the different plasmid profiles, another 100 transconjugants were selected, restreaked and analyzed for plasmid DNA (Table 8).

The two largest plasmids of R. trifolii strain T37, pRtT37b and pRtT37c were present in all transconjugants with no observable differences in electrophoretic mobility in agarose gels.

Approximately 30% of the transconjugants (Class I) contained a 130 Mdal plasmid corresponding in size to pJB5JI (Figure 18, lane 3). These transconjugant strains lacked a plasmid band corresponding to pRtT37a. Another 40% of the transconjugant strains (Class II) lacked a plasmid corresponding to pJB5JI. These transconjugants contained plasmids ranging in size from 140 Mdal to approximately 270 Mdal (Figure 18, lanes 4-7). The remaining 30% of the transconjugants (Class III) did not contain plasmids corresponding to pJB5JI or pRtT37a nor were plasmids of molecular weight intermediate to pJB5JI and pRtT37a observed (Figure 18, lane 8). Four transconjugants (Class IV) were obtained which harbored two plasmids with molecular weights intermediate to that of pRtT37a and pJB5JI (Figure 18, lanes 9 and 10). In addition, the smallest plasmid of the donor strain, pR1T83K3a, randomly cotransferred with pJB5JI into approximately 33% of the transconjugants of each class. No additional plasmid rearrangements were observed in the isolates harboring this plasmid.

Symbiotic Properties of R. trifolii Strain T37 (pJB5JI) Transconjugants. The symbiotic properties of strains from each class were examined by inoculation of white clover plants, the normal R. trifolii host, and pea plants, the host plant specified by pJB5JI-encoded genes. Differences in N_2-fixation (Fix) and nodulation abilities (Nod) of the transconjugant stains were evident upon examination of both plant

Table 8. Diagrammatic representation of plasmids of strain T37 (pJB5JI) transconjugants after agarose gel electrophoresis.

Class I	Class II				Class III	Class IV	
——	——	——	——	——	——	——	——
——	——	——	——	——	——	——	——
				——		——	——
	——	——	——			——	——
——							
---[a]	---	---	---	---	---	---	---
6016	6024*	6012	6045	6001	6002	6030	6088
6017*	6041		6063	6003	6005*	6135*	6137
6018*	6046		6076*	6004	6006*		
6022*	6060		6084	6020*	6007		
6027*	6074*		6106	6025*	6008*		
6032	6090*			6047	6009		
6038	6109*			6049*	6010*		
6042*	6111			6051*	6014		
6043*	6131*			6058*	6015		
6044*	6133			6059*	6021		
6048*	6134			6062*	6023		
6052*				6064*	6026		
6054				6065*	6028		
6055				6066*	6033*		
6069				6067	6034		
6070				6075	6036		
6073*				6088*	6037*		
6078				6092	6039*		
6080				6093	6057*		
6082*				6096	6061		
6086				6101	6068*		
6087*				6102	6071		
6091				6103	6072		
6095				6104*	6077		
6097*				6107*	6079		
6099*				6108*	6081		
6100*				6110	6083		
6105				6113	6085		
6117				6115	6089*		
6126*				6116	6094		
6132				6119*	6098*		
6138*				6120	6112		
				6121	6114*		
				6122*	6118		
				6124	6123		
				6127*	6125		
				6128	6130*		
				6129	6136*		
					6140		

[a]Dashed lines represent pR1T83K3a which was present only in strains designated with an asterisk.

hosts (Table 9). Transconjugant strains in Class I were unable to nodulate clover plants while all the transconjugants of Class II and III (except strain 6023) formed effective (Fix^+) nodules on clover roots. Nodulation of clover plants by the transconjugant strains was not delayed when compared to the wild-type R. trifolii strain T37.

All transconjugants strains tested from the three classes nodulated peas (Table 9). All transconjugants in Class I and 5 out of 6 transconjugants in Class III formed pink, effective (Fix^+) nodules on pea plants. Nodules produced from Class II transconjugants were white and were ineffective (Fix^-).

Bacteria were isolated from the root nodules of pea and clover plants and analyzed for plasmid DNA. The nodule isolates were found to have the same plasmid profiles as the transconjugant strains used as inocula (Figure 19). In addition, bacteria isolated from pea root nodules were used to inoculate clover plants. No change in the expected phenotype was observed. Similar results were obtained from clover nodule isolates used to inoculate pea plants.

Thus, the plasmid alterations occurring upon transfer of pJB5JI to R. trifolii strain T37 were stable, even after passage of the transconjugant strains through root nodules on both host plants.

Fahraeus Slide Analysis of Infection Process. The infection process of three Nod^- transconjugant strains on white clover root hairs was examined microscopically using the Fahraeus slide technique (65). The three Nod^- transconjugant strains were: Class I strains 6032 and 6017, and Class III strain 6023. R. trifolii strain T37 and R. leguminosarum strain T83K3 were used as positive and negative controls, respectively.

Table 9. Nodulation and nitrogen fixation abilities of the four classes of transconjugant strains on white clover and peas.

Class	White Clover		Pea	
	No. Nod^{+a} / No. tested	No. Fix^{+b} / No. Nod^{+}	No. Nod^{+} / No. tested	No. Fix^{+} / No. Nod^{+}
Class I	0/10	-	6/6	6/6
Class II	10/10	10/10	6/6	0/6
Class III	9/10[c]	9/9	6/6	5/6[d]
Class IV	3/3	3/3	3/3	3/3

Plants were grown as described in Materials and Methods and were examined for nodules and tested for acetylene reduction ability after five weeks. No nodules were observed on peas inoculated with *R. trifolii* strain T37, clover plants inoculated with *R. leguminosarum* strain T83K3, or uninoculated plants.

[a]The number of transconjugants which formed nodules on clover plants out of the number tested.
[b]The fraction of transconjugants which formed nodules that actively reduced acetylene.
[c]*R. trifolii* strain 6023 did not nodulate clover but formed effective nodules on peas.
[d]Strain 6130 formed ineffective nodules on peas and effective nodules on clover.

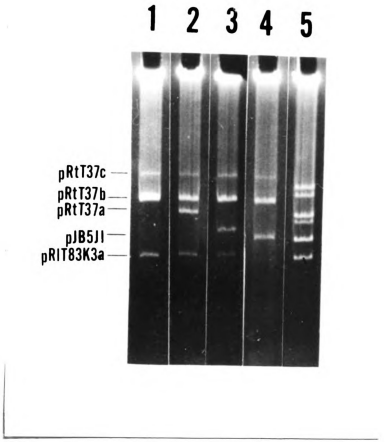


Figure 19. Ekhardt agarose gel electrophoresis of bacteria isolated from pea nodules. Lane 1, Class III strain 6130; 2, Class II strain 6049; 3, Class II strain 6131; Class I strain 6080; 4, R. leguminosarum strain T83K3.

Bacterial cells from all of the cultures used to inoculate white clover plants bound to the tips of clover root hairs (Figure 20, c and d). Slight curling or deformation and some branching of root hairs were observed in plants inoculated with transconjugant strains 6032 and 6017, and R. leguminosarum strain T83K3. In general, though, the root hairs were relatively unaffected (Figure 20,c).

In contrast, marked curling of clover root hairs was observed on plants inoculated with the Nod$^-$ transconjugant strain 6023 and with R. trifolii strain T37 (Figure 20, a and b). Infection threads were observed to have formed four days after inoculation of the clover plants with R. trifolii strain T37 (Figure 21). Even though root hairs of plants inoculated with strain 6023 were markedly curled, no infection threads were observed. This suggests that DNA coding for an essential step in the formation of infection threads has been deleted in strain 6023.

The response observed for plants inoculated with strain 6032 and 6017 was very similar to that obtained when R. leguminosarum strain T83K3 was used as the inoculum. The absence of marked curling of root hairs and of the development of infection threads in plants inoculated with these two transconjugant strains indicated that DNA encoding genes essential for these processes had been deleted from these strains.

Tn 5 Hybridization Analysis. The absence of a plasmid corresponding in size to pJB5JI in the Class II and Class III transconjugant strains indicated a possible loss of pJB5JI DNA. The selection was based on resistance to kanamycin, however, which suggests that Tn 5 may have transposed from pJB5JI to a new site in the R. trifolii genome. Transposition of Tn 5 would result in new restriction endonucleases

Figure 20. Fahraeus slide analysis of root hairs of clover plants inoculated with strains of Rhizobium. (a) R. trifolii strain T37; (b), Class III strain 6023; (c) Class I strain 6032; (d) R. leguminosarum strain T83K3.

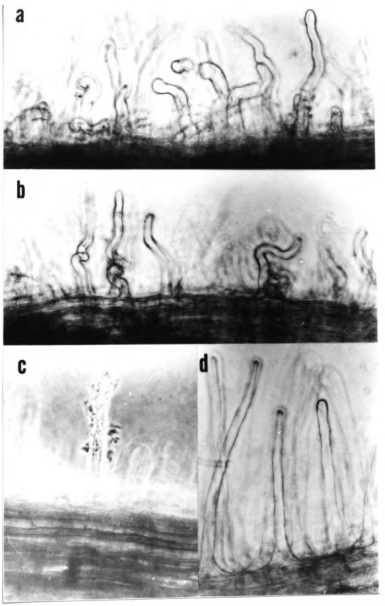
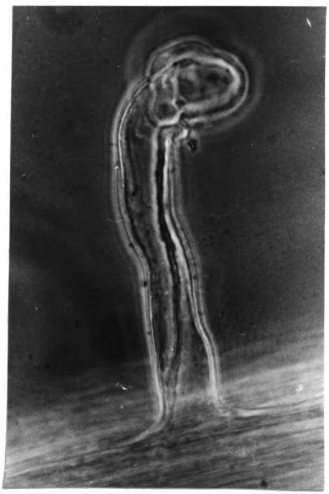

a
b
c
d

Figure 21. Infection thread in a root hair of a clover plant inoculated with R. trifolii strain T37.



sites in the sequences flanking Tn 5 DNA. To investigate this, the flanking regions of Tn 5 DNA were characterized for several transconjugant strains. A partial restriction map of Tn 5 DNA is shown in Figure 22. Tn 5 does not contain an Eco RI restriction endonuclease cleavage site. Thus, Eco RI-digested total DNA from the transconjugant strains should contain only one Eco RI fragment that will hybridize to ^{32}P-labeled Tn 5 probe. If Tn 5 has transposed, the fragment containing Tn 5 would be different in molecular weight. A single hybridization band of approximately 11 kilobases (kb) was observed in an autoradiogram of a Southern filter of Eco RI-digested total DNA from donor, recipient, and several transconjugant strains probed with ^{32}P-labeled λ::Tn 5 DNA (Figure 23). No hybridization was observed to the recipient strain DNA (Figure 23, lane 7). To further confirm that Tn 5 had not transposed from pJB5JI DNA, total DNA from the transconjugants was digested with Bgl II restriction endonuclease and analyzed by Southern hybridization. Tn 5 is cleaved twice by Bgl II, once each in the inverted terminal repeats of the transposon. Thus, three Bgl II fragments should hybridize to the Tn 5 probe. Hybridization to 9.6, 2.65, and 2.5 kb fragments in the Bgl II-digested DNA from the donor and several transconjugant strains was observed (Figure 23).

The hybridization data above indicated that Tn 5 had not transposed from pJB5JI. Since pJB5JI transfers at high frequency and no conjugative plasmids have been observed in R. trifolii strain T37, the correlation of the transfer of kanamycin resistance with the transfer of a specific plasmid from the strain T37 (pJB5JI) transconjugants to R. leguminosarum strain 726 would indicate which plasmid contained Tn5, and thus pJB5JI DNA. Therefore, the transfer of kanamycin resistance

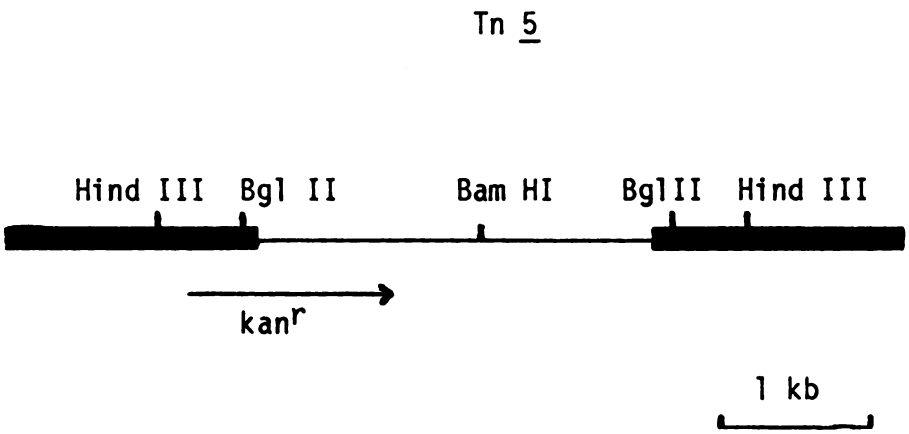


Figure 22. Partial restriction map of Tn 5. Heavy lines indicate inverted repeats. Coding region for kanamycin resistance is indicated by the arrow. Data were obtained from Jorgensen et al. (102).

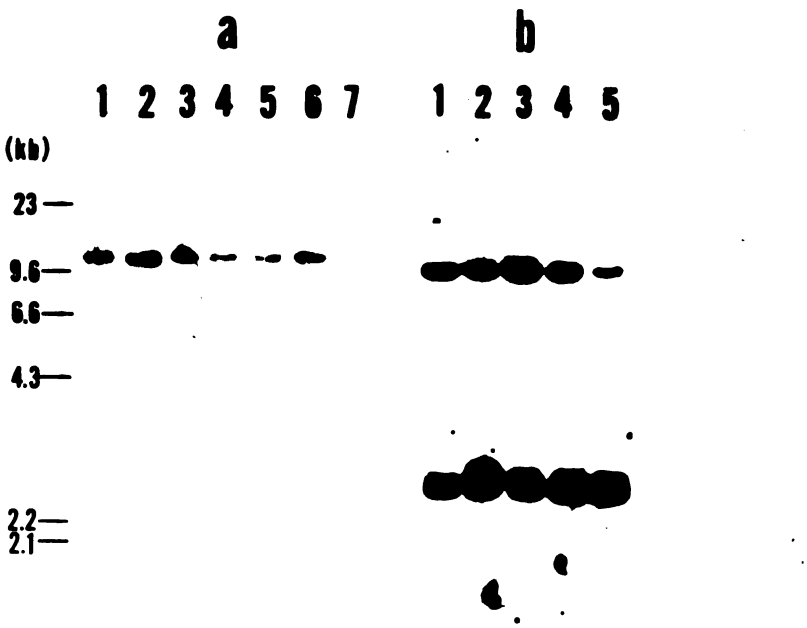


Figure 23. Autoradiogram of ^{32}P-labeled λ::Tn 5 DNA hybridized to restriction endonuclease-digested DNA. Panel a, Eco RI-digested DNA from: (1), R. trifolii strain 6002; (2), strain 6032, (3), strain 6067; (4), strain 6007; (5), R. leguminosarum strain T83K3; (6), strain 6012; (7), R. trifolii strain T37. Panel b, Bgl II-digested DNA from: (1), R. trifolii 6032; (2), R. trifolii 6093; (3), R. leguminosarum T83K3; (4), R. trifolii 6023; (5), R. trifolii 6012.

from a number of strain T37 (pJB5JI) transconjugants to R. leguminosarum strain 726 was determined. Plasmid profiles of the resulting transconjugants were also analyzed.

The transfer frequencies varied from the normal value observed for pJB5JI of approximately 10^{-3} for the transconjugant strains of Class I, to very low values (10^{-6} to 10^{-7}) observed for some strains (Table 10).

Several transconjugants from each cross were restreaked and analyzed for plasmid DNA (Figure 24). The 130 Mdal plasmid corresponding to pJB5JI transferred to R. leguminosarum strain 726 from Class I strains 6032 and 6069. The fluorescence of the fourth smallest plasmid (the smallest plasmid is a doublet as in R. leguminosarum strain T83K3) of transconjugants from the latter crosses was more intense in EtBr-stained Ekhardt agarose gels than the corresponding plasmid in gels of transconjugants obtained from other crosses (Figure 24, lane 5). This suggested that a plasmid of the same molecular weight (about 300×10^6) had transferred from the Class III strains into R. leguminosarum strain 726.

The presence of Tn 5 on these plasmids was confirmed by hybridization of ^{32}P-labeled λ::Tn 5 DNA to a Southern filter of an Ekhardt gel similar to the one shown in Figure 18. The Tn 5 probe hybridized to pJB5JI in the donor strain and to a plasmid of the same molecular weight in Class I transconjugants (Figure 25; lanes a,c). In Class II transconjugants, Tn 5 hybridized to the plasmid ranging in molecular size between those of pBJ5JI and pRtT37b (lanes d-g). The Tn 5 probe hybridized to plasmid DNA in the region of pRtT37b in Class III transconjugants (lane h).

Table 10. Frequency of transfer of kanamycin resistance from R. trifolii strain T37 (pJB5JI) transconjugants to R. leguminosarum strain 726

Class	Donor Strain	Kanamycin Resistance Transfer Frequency
I	6032	9.0×10^{-4}
	6055	2.1×10^{-3}
	6070	2.9×10^{-3}
	6017*	1.3×10^{-3}
	6022*	7.2×10^{-4}
	6044*	3.1×10^{-3}
II	6012	1.3×10^{-5}
	6060	1.2×10^{-7}
	6067	3.1×10^{-3}
	6076	1.4×10^{-3}
	6092	4.1×10^{-4}
	6049*	8.1×10^{-5}
	6059*	2.3×10^{-3}
	6064*	2.2×10^{-4}
	6074*	3.8×10^{-7}
	6109*	4.0×10^{-4}
III	6002	2.8×10^{-4}
	6007	2.0×10^{-4}
	6023	1.4×10^{-4}
	6005*	9.2×10^{-6}
	6033*	1.3×10^{-7}
	6114*	4.7×10^{-6}

Transconjugants were selected on 40 µg/ml rifampicin and 200 µg/ml kanamycin. The transfer frequency was determined per recipient cell plated. The frequency of spontaneous resistance of the donor strains to 40 µg/ml rifampicin and of the recipient strain to 200 µg/ml kanamycin was 1.5×10^{-8} and $< 6 \times 10^{-9}$, respectively.

*These strains also contain pR1T83K3a.

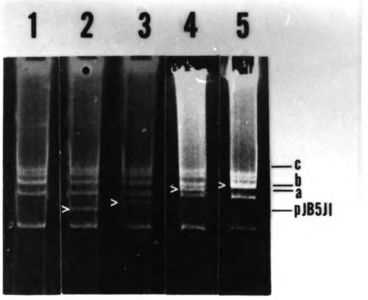


Figure 24. Ekhardt agarose gel electrophoresis of isolates of bacterial crosses of R. trifolii strain T37 (pJB5JI) transconjugants with R. leguminosarum strain 726. Lane 1, R. leguminosarum strain 726 (recipient); lanes 2-5, transconjugants from crosses with Class I strain 6032, Class II strain 6060, Class II strain 6067, and Class III strain 6023, respectively. The plasmid transferred is indicated by >.

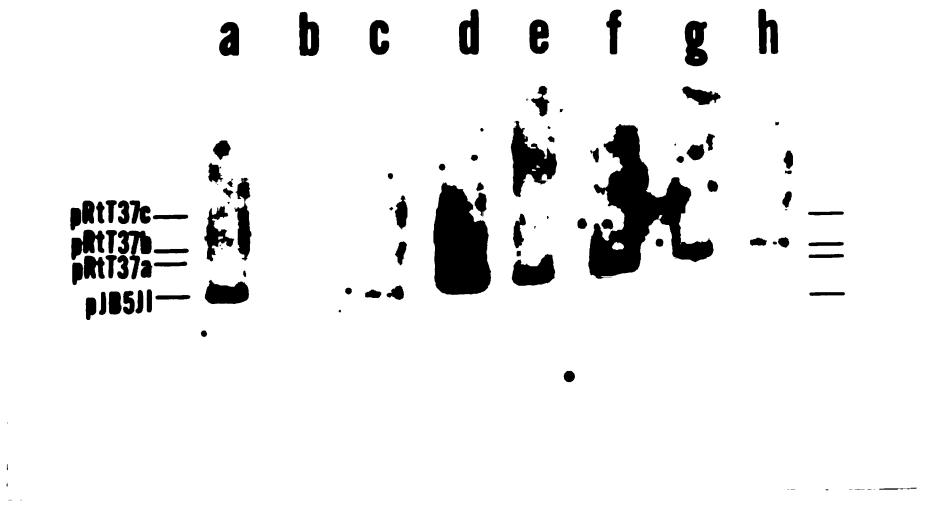


Figure 25. Autoradiogram of ^{32}P-labeled λ::Tn 5 DNA hybridized to a Southern filter of an Ekhardt agarose gel. Lane a, R. leguminosarum strain T83K3; (b), R. trifolii strain T37; (c), Class I strain 6032, (d-g), Class II strains 6060, 6012, 6076, 6067, respectively; (h), Class III strain 6130. The positions of certain plasmids are noted.

Tn 5 was also located on a specific plasmid in the Class IV transconjugants strains (Figure 26). The Tn 5 probe hybridized to the smallest of the two plasmids of molecular weight intermediate to the molecular weights of pJB5JI and pRtT37a in strains 6135 and 6137.

nif Hybridization Analysis. Plasmids containing the nif structural genes were also identified in the donor, recipient, and transconjugant strains. Southern blot analysis of an Ekhardt agarose gel probed with ^{32}P-labeled R. meliloti nif DNA showed hybridization with pJB5JI and pRtT37a (Figure 27), lanes a and b). The plasmids in Class I, II, and III transconjugant strains which hybridized with Tn 5 also hybridized with the nif DNA probe (Figure 26, lanes c-h). The nif probe hybridized with the higher molecular weight "hybrid" plasmid in Class IV transconjugant strains 6135 and 6137 (Figure 26).

The R. meliloti nif DNA is homologous to both R. leguminosarum and R. trifolii nif DNA. Thus, it was not possible to distinguish between the presence in the transconjugants of nif DNA from only one strain or both R. trifolii and R. leguminosarum. To resolve this, total DNA from R. trifolii strain T37 and R. leguminosarum strain T83K3 was digested with restriction enzymes and examined for the presence of the nif structural genes by Southern blot analysis. The R. trifolii strain T37 nif structural genes were located on a 5.0 kb Eco RI fragment (Figure 28, lane e), while the nif genes (located on pJB5JI) in R. leguminosarum strain T83K3 were present on a 2.65 kb Eco RI fragment (Figure 28, lane a). This gave an easy method of distinguishing between the nif DNA of R. trifolii strain T37 and R. leguminosarum strain T83K3 and therefore, which nif DNA was present in the transconjugant strains. Eco RI-digested DNA from transconjugant strains was examined in the

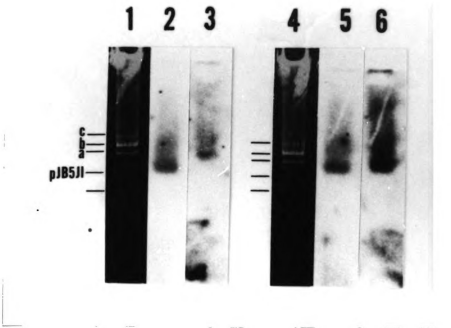


Figure 26. Hybridization analysis of plasmids of Class IV transconjugant strains 6135 and 6137. Lane 1, EtBr-stained plasmid DNA in strain 6137; Lanes 2 and 3, Southern filters of the gel in lane 1 probed with ^{32}P-labeled λ::Tn *5* (lane 2) or ^{32}P-labeled *R. meliloti nif* DNA (lane 3). Lane 4, EtBr-stained plasmid DNA in strain 6135; lanes 5 and 6, Southern filters of the gel in lane 4 probed with ^{32}P-labeled λ::Tn *5* DNA (lane 5) or ^{32}P-labeled *R. meliloti nif* DNA (lane 6).

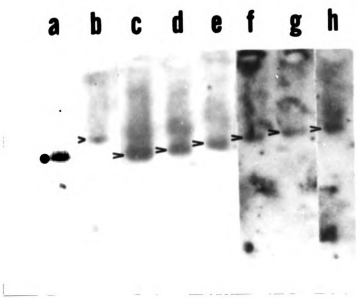


Figure 27. Autoradiogram of ^{32}P-labeled R. meliloti nif DNA hybridized to Southern filters of an Ekhardt agarose gel similar to Figure 13. Lane a, R. leguminosarum strain T83K3; (b), R. trifolii strain T37; (c), Class I strain 6032; (d-g), Class II strains 6060, 6012, 6076, 6067, respectively; (h), Class III strain 6130. The mobilities of the three R. trifolii strain T37 plasmids and pJB5JI are indicated at the right and left of the autoradiogram. (0), pJB5JI; (>), pRtT37a; (Δ) transconjugant plasmids encoding nif DNA.

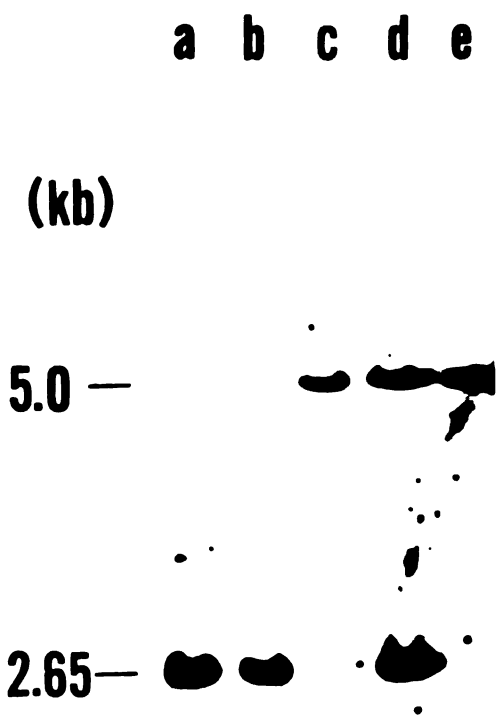


Figure 28. Autoradiogram of ^{32}P-labeled K. pneumoniae nif DNA hybridized to Southern blots of Eco RI-digested DNA from Rhizobium strains. (a), R. leguminosarum strain T83K3; (b), Class I strain 6032; (c), Class II strain 6012; (d), Class III strain 6007; (e), R. trifolii strain T37.

same manner. Three patterns of hybridization of the nif probe to Eco RI-digested DNA from transconjugants were observed. The nif probe hybridized only to a 2.65 kb Eco RI fragment of DNA from Class I transconjugants (Figure 28, lane b). This fragment corresponded to the R. leguminosarum nif-containing DNA fragment. In Class II transconjugants, only a 5.0 kb fragment corresponding to the R. trifolii strain T37 nif DNA hybridized (Figure 28, lane c), whereas, both fragments hybridized to the nif probe in Class III strains (Figure 28, lane d). Similar analysis of DNA from the Class IV strains 6088, 6135, and 6137 indicated that both R. leguminosarum and R. trifolii nif DNA was present as noted for Class III strains (data not shown). Since only one plasmid in these strains hybridized to the nif DNA probe, both the R. trifolii and the R. leguminosarum nif DNA must be on the same "hybrid" plasmid. In strains 6088 and 6137, this plasmid did not hybridize to the Tn 5 probe, a further indication that recombination between pJB5JI and pRtT37a had occurred.

pJB5JI and pRt737a Hybridization Studies. In order to determine the amount of pJB5JI and pRtT37a still present in the transconjugant strains, the plasmids were isolated, labeled in vitro, and hybridized to Southern transfers of Eco RI-digested total DNA from several strains from each class of transconjugants.

Initially, pJB5JI was isolated by the alkaline lysis procedure and purified on CsCl-EtBr density equilibrium gradients. Plasmid DNA purified in this manner would be free of chromosomal DNA, but would also contain pR1T83K3a/b DNA and, to a lesser extent, the higher molecular weight plasmids of strain T83K3. The presence of pR1T83K3 a/b DNA

would not affect the hybridization results because total DNA was isolated from transconjugant strains which did not contain pRlT83K3a.

Hybridization of pJB5JI DNA purified by CsCl-EtBr gradient centrifugation to a Southern filter of Eco RI-digested total DNA from several transconjugant strains is shown in Figure 29. The hybridization pattern was complex due to the large number of restriction fragments which are homologous to pJB5JI. Basically the same pattern of hybridization was observed in all of the strains examined. Several differences were detected, however. Hybridization to 4.3 kb and 6.0 kb Eco RI restriction fragments was not observed in Class I transconjugant DNA but was observed to these fragments in the DNA of Class II strains (Figure 29). This indicates that some pJB5JI sequences have been deleted from Class I strains.

No major differences in the hybridization pattern were observed for Class II transconjugants which contained "hybrid" plasmids of various molecular weights. This indicated that a similar amount of pJB5JI DNA was present in these transconjugant strains. Hybridization of ^{32}P-labeled pJB5JI to Eco RI-digested DNA from R. leguminosarum strain T83K3 was not carried out due to the presence of pRlT83K3a/b in both the probe and in strain T83K3 DNA. This would have allowed the determination of the number of Eco RI restriction fragments in pJB5JI and thus whether some pJB5JI sequences had been deleted from the strains examined.

To address this problem, and to determine the amount of pRtT37a which is present in the transconjugant strains, hybridization to Eco RI digested total DNA from transconjugant strains was carried out with pJB5JI and pRtT37a isolated from low-melting-temperature agarose gels

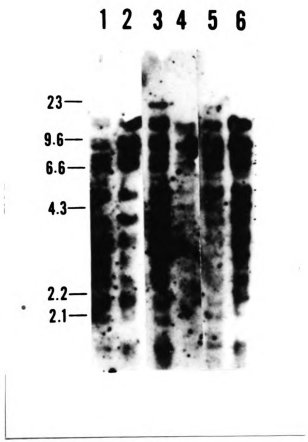


Figure 29. Autoradiogram of ^{32}P-labeled pJB5JI DNA hybridized to a Southern filter of Eco RI-digested total DNA from transconjugant strains. The pJB5JI DNA was isolated by CsCl·EtBr density equilibrium centrifugation and ^{32}P-labeled in vitro by the nick translation method. Lane 1, Class I strain 6032; 2, Class I strain 6069, 3-6, Class II strains 6084, 6060, 6012, 6093, respectively.

as described in the Methods. Recovery of plasmid DNA from agarose gels allowed isolation of a specific plasmid, unlike CsCl-EtBr gradients where total, supercoiled plasmid DNA was isolated.

The results of hybridization of ^{32}P-labeled pJB5JI to Eco RI-digested DNA from donor, recipient and transconjugant DNA are shown in Figure 30. In general, less hybridization was observed to DNA from the transconjugants than to R. leguminosarum T83K3 (positive control). Differences in the pattern of hybridization of pJB5JI to DNA from Classes I, II, and III were also observed. Thus, varying amounts of pJB5JI appear to have been deleted during the recombination event with pRtT37a. No hybridization was observed to DNA from R. trifolii strain T37 under these exposure conditions. However, exposure of the film to the filter for about 7 days allowed detection of Eco RI restriction fragments of R. trifolii strain T37 which were homologous to pJB5JI (Figure 31).

Hybridization of ^{32}P-labeled pRtT37a to Southern filters of Eco RI-digested transconjugant DNA was carried out in a similar manner (Figure 32). A large number of fragments in the transconjugant strains were homologous to pRtT37a, but the amount of pRtT37a present in the strains could not be determined quantitatively due to the complexity of the hybridization pattern and the high background. To further complicate the interpretation of the results, pRtT37a hybridized to R. leguminosarum strain T83K3 DNA (Figure 32, lane 1). These sequences are quite homologous, since the filters were washed under stringent conditions (0.1X SSC, 68°C). Due to the complexity of pRtT37a probe, long exposures of the film to the Southern filters were necessary for the detection of restriction fragments of pRtT37a. These long

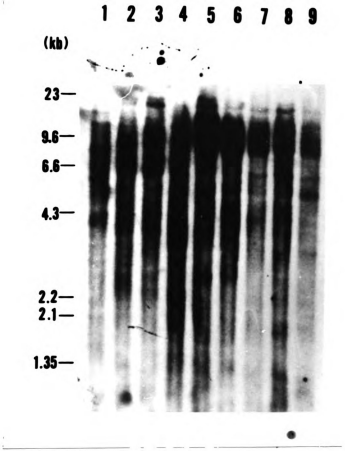


Figure 30. Autoradiogram of ^{32}P-labeled pJB5JI DNA to a Southern filter of Eco RI-digested total DNA from donor, recipient and transconjugants. The pJB5JI DNA was isolated from low-melting-temperature agarose gels and ^{32}P-labeled in vitro by the nick translation method. Lane 1, R. leguminosarum strain T83K3 (donor); (2), Class I strain 6032; (3-6), Class II strains 6060, 6012, 6076, and 6067, respectively; (7-8), Class III strains 6002 and 6023, respectively; (9), R. trifolii strain T37 (recipient).

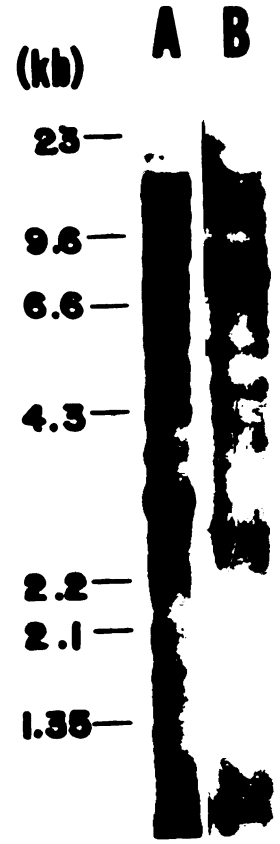


Figure 31. Autoradiogram of ^{32}P-labeled pJB5JI DNA hybridized to a Southern filter of Eco RI-digested DNA from donor and recipient strains. A, R. leguminosarum strain T83K3 (5 µg); B, R. trifolii strain T37 (5 µg). Film was exposed for 12 h (A) or 7 days (B).

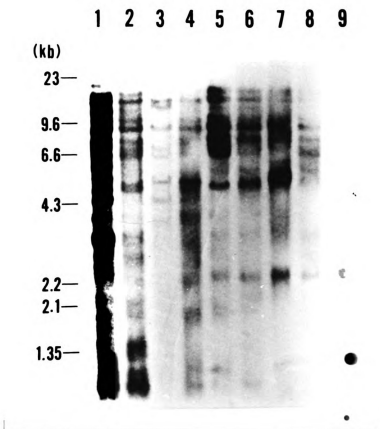


Figure 32. Autoradiogram of ^{32}P-labeled pRtT37a DNA hybridized to a Southern filter of Eco RI-digested DNA from donor, recipient and transconjugant strains. Lane 1, R. leguminosarum strain T83K3 (donor); (2), Class I strain 6032; (3-7), Class II strains 6060, 6012, 6067, 6086, and 6093, respectively; (8), Class III strain 6023; (9), R. trifolii strain T37 (recipient).

exposures, however, allowed detection of the fragments of pJB5JI which are homologous to pRtT37a. Thus, the varying amounts of pRtT37a present in the transconjugant strains could not be determined.

DNA from Class I transconjugant strain 6032 which contained a plasmid of molecular weight similar to that of pJB5JI, hybridized with ^{32}P-labeled pRtT37a to a greater extent than the DNA in the donor strain (Figure 32, lanes 1 and 2). Thus, the plasmid in Class I strains which corresponded in molecular weight to pJB5JI contained some DNA from pRtT37a.

DISCUSSION

At least three large plasmids were present in R. trifolii strain T37; four large plasmids were observed in R. trifolii strain 0403. Transfer of the conjugative R. leguminosarum plasmid pJB5JI into R. trifolii strain 0403 did not alter the plasmid profile of the four resident plasmids of this strain as determined by Ekhardt agarose gel electrophoresis. In contrast, dramatic changes in the plasmid profile were evident upon transfer of pJB5JI into R. trifolii strain T37. The observed changes in the plasmid profiles of the strain T37 (pJB5JI) transconjugants apparently resulted from the recombination between pJB5JI and pRtT37a. The hybrid plasmids which resulted from this recombination event contained variable amounts of pJB5JI and pRtT37a based on an analysis of the symbiotic properties of the transconjugants on both clover and peas and on hybridization studies using ^{32}P-labeled λ::Tn 5 and heterologous nif DNA.

Johnston et al. (100) reported that transconjugants resulting from the transfer of pJB5JI into heterologous species of Rhizobium retained the ability to nodulate their normal host, although fewer nodules were formed and they were slower to appear. These transconjugants also nodulated pea plants but the nodules produced were not always effective. The presence of genetic information necessary for the nodulation of a second host plant in a Rhizobium strain was suggested to impair the nodulation of the normal host plant.

The symbiotic properties of R. trifolii strain 0403 (pJB5JI) transconjugants on the normal host plant, white clover, were not altered. The host range of these transconjugants was extended, however, to include pea plants as a result of pJB5JI transfer. The root nodules induced on peas by these transconjugants were unable to reduce acetylene (Fix^-) which was similar to results obtained for other strains of Rhizobium into which pJB5JI had been transferred (100). A derivative of pJB5JI with a 30 Mdal deletion was also observed in one transconjugant. This transconjugant was unable to nodulate peas suggesting that the 30 Mdal region of deleted DNA contained gene(s) essential for pea nodulation. Southern hybridization analysis demonstrated that the R. leguminosarum nif structural genes had also been deleted (Figure 17). Spontaneous deletion of pJB5JI DNA resulting in the loss of pea nodulation ability has been observed previously in pJB5JI transfers (56).

The R. trifolii strain T37 (pJB5JI) transconjugants obtained in this study varied in their symbiotic properties on the two host plants, pea and white clover. Class I transconjugants were missing a plasmid corresponding in size to pRtT37a and did not nodulate white clover plants. Little curling or deformation of root hairs of clover plants inoculated with two Class I transconjugant strains (strain 6032 and 6017) was observed (Figure 20), suggesting that gene(s) encoding the curling inducer(s) had been deleted during the recombination event. In contrast, strain 6023, a Nod^- Class III strain, induced "marked" curling of root hairs (Figure 20). Class II, Class III, and Class IV transconjugants contained "hybrid" plasmids and formed effective nodules on clover. No delay in the nodulation of clover plants by Class

II and III transconjugants as compared to wild-type strain T37 was observed. The *R. trifolii nif* genes were missing in Class I strains but were present in Classes II, III, and IV transconjugants. These findings suggest that pRtT37a codes for genes essential for nodulation of clover as well as the *R. trifolii nif* genes and that the hybrid plasmids in strains of all four classes of transconjugants contain some of pRtT37a.

All four classes of *R. trifolii* T37 (pJB5JI) transconjugants nodulated peas although nodulation was delayed several days and fewer nodules were formed than the number induced by *R. leguminosarum* T83K3, the pJB5JI donor strain. All three classes contained *nif* genes but only Class I, Class III, and Class IV strains (which contained *R. leguminosarum nif* genes) formed effective nodules on peas. Nodules induced by Class II transconjugants which lacked the *R. leguminosarum nif* genes were white and ineffective. Although the *R. trifolii nif* genes were present in these transconjugants and were functional in clover nodules, they did not complement the deleted *R. leguminosarum nif* genes in pea nodules.

There are several possible explanations for this apparent anomaly. The biochemical signal necessary for activating the expression of the *R. leguminosarum nif* genes in pea nodules may be different from that required for expression of the *R. trifolii nif* genes in clover nodules. If true, the *R. trifolii nif* genes may not be expressed in pea nodules and this could account for the *Fix*$^-$ phenotype of this class of transconjugants on peas. Alternatively, the *R. trifolii nif* genes may be transcribed and translated in pea nodules but may not lead to active gene products. Possibly these transconjugants lack essential factors

which fail to allow the recognition of signals from pea plants which would permit differentiation of the bacteria into functional bacteroids in pea nodules. Another explanation is that these transconjugants lack factors which would elicit the expression of plant proteins, such as apoleghemoglobin, which are essential for an effective symbiosis. These factors could be encoded on parts of pJB5JI deleted during formation of the hybrid plasmids or on parts of the R. leguminosarum genome not transferred to strain T37. The latter is unlikely since Class I and III transconjugants form effective nodules on peas.

A "switch mechanism" was postulated to explain the instability of the Fix^+ phenotype of R. trifolii strains into which pJB5JI had been transferred (56). Vacillation between Fix^+ and Fix^- phenotypes on pea and clover plants was observed. No changes in the indigenous R. trifolii plasmids or recombination events between pJB5JI and the R. trifolii plasmids were reported.

In contrast, we have observed recombination between pRtT37a and pJB5JI which resulted in alterations in the plasmid profiles of the transconjugant strains. The recombination resulted in the deletion of either the R. trifolii nif and nod genes (Class I) or the R. leguminosarum nif genes (Class II). In Class III and Class IV transconjugant strains, both sets of sym genes were stably maintained and were functional on both host plants. No oscillation between Fix^+/Fix^- phenotypes was observed.

The recombination event between pJB5JI and pRtT37a may occur by several mechanisms. The altered plasmid profiles may result from recombination between homologous regions of DNA on the two plasmids, in which varying amounts of plasmid DNA are deleted. Prakash et al. (147)

using non-stringent hybridization and washing conditions, observed extensive DNA homology between nif-containing plasmids of R. leguminosarum, R. phaseoli, and R. trifolii. Conservation of DNA on nif-containing plasmids in these strains of Rhizobium was proposed.

In fact, there are regions of homology on pJB5JI and pRtT37a. Both plasmids hybridize to ^{32}P-labeled nif DNA (Figure 27, a and b). However, between species, the homologous region of nif DNA is only about 1.5 kb (166). Pea nodulation genes are encoded on pJB5JI (100) and clover nod genes have been reported to be plasmid encoded in R. trifolii (95,215). Results presented here suggest that genes essential for clover nodulation are encoded on pRtT37a. The degree of homology between pea and clover nod genes is not known.

Homology between pJB5JI DNA and pRtT37a DNA was observed even under stringent hybridization and washing conditions (Figure 31; Figure 32, lane 1). This region of DNA common to both plasmids may have allowed recombination to occur. However, recombination between pJB5JI and plasmids of R. trifolii strain 0403 or other strains has not been observed (Figure 16, reference 56). Thus, if the recombination event occurs by this mechanism, the DNA sequences involved must be unique to pRtT37a and not other R. trifolii plasmids.

Another mechanism for the recombination between pJB5JI and pRtT37a may be the action of transposable elements and/or insertion sequences. The formation of cointegrates has been proposed as an intermediate step in the transposition and replication of transposable elements and insertion sequences (33,181). Evidence reported herein suggests, however, that the recombination is not mediated by Tn 5 transposition. Southern blot analysis of donor and transconjugant DNA flanking Tn 5

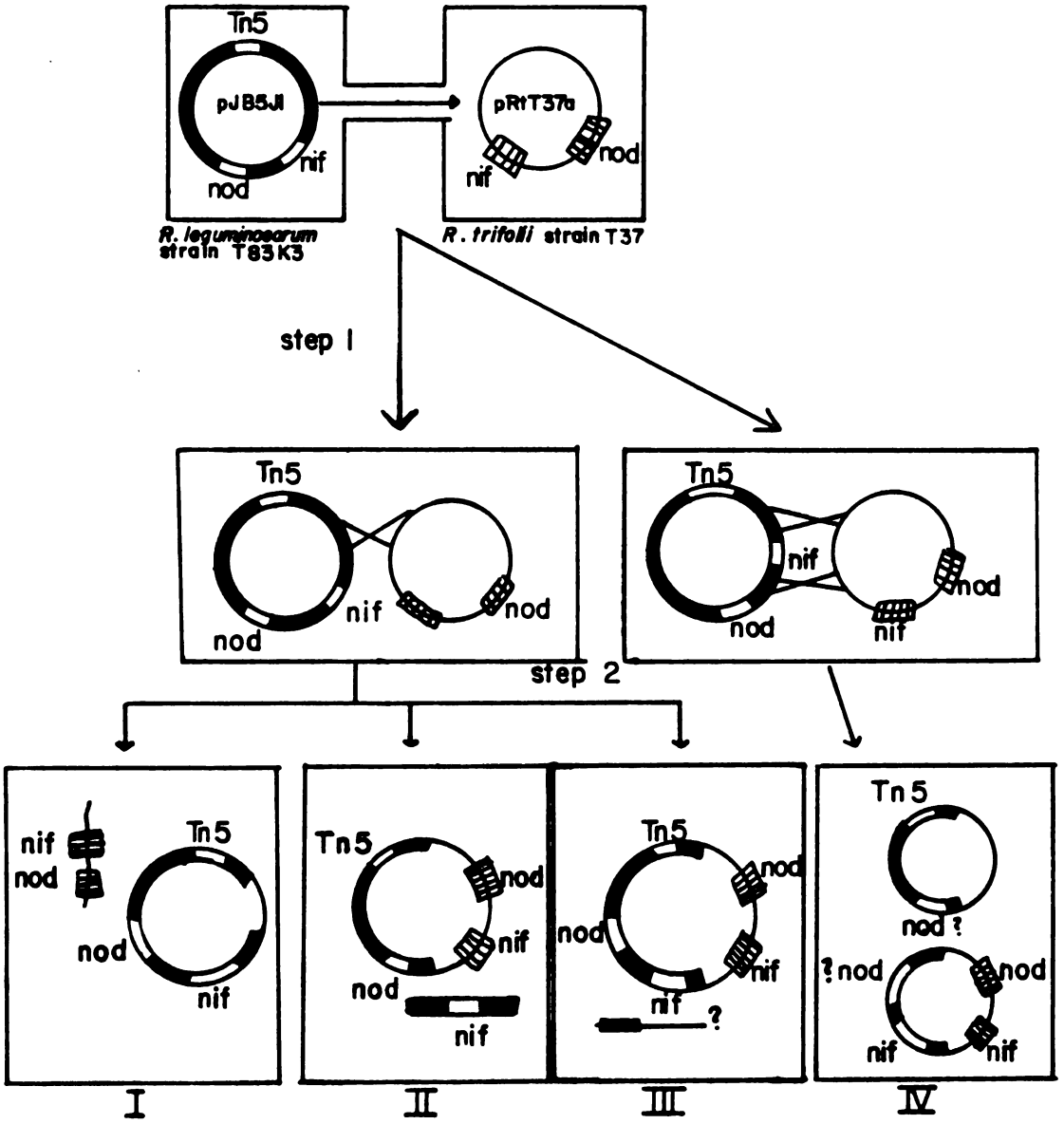
demonstrated that Tn 5 had not transposed (Figure 23). Also genetic analysis of Tn 5 transposition in R. leguminosarum (12) and R. meliloti (126) indicated that transposition occurs only at very low frequency (approximately 10^{-8}) in Rhizobium. Indigenous insertion elements have been identified in R. lupini (149) and in one strain of R. meliloti (167), but have not as yet been reported in R. trifolii.

A model based on the recombination between homologous regions of pJB5JI and pRtT37a is shown in Figure 33. Conjugal transfer of pJB5JI from R. leguminosarum strain T83K3 to R. trifolii strain T37 occurs first (step 1). This is followed by recombination between pJB5JI and pRtT37a (step 2), accompanied by the deletion of varying amounts of plasmid DNA. In some cases (Class I and II) nod and/or nif genes are deleted.

Class IV strains, which contain two "hybrid" plasmids, could arise by a double crossover between pJB5JI and pRtT37a. During this event, the R. leguminosarum nif genes (and possibly the nod genes) would be recombined into pRtT37a. This would explain the hybridization data in which Tn 5 was localized on a different plasmid than the nif genes (Figure 26). The plasmid in Class IV strains on which the pea nod genes were located was not determined. The nif and nod genes have been shown to be linked on pJB5JI (94) and also on plasmid in other strains of Rhizobium (5,164). This suggests that the pea nod genes may be located on the same plasmid as the nif genes.

This model indicates how the plasmids observed in the transconjugant strains could result, and explains the Tn 5 and nif hybridization data and also the observed symbiotic phenotypes of these strains inoculated on pea and clover plants.

Figure 33. A model for the generation of the "hybrid plasmids of R. trifolii strain T37 (pJB5JI) transconjugants. Step 1, transfer of pJB5JI from R. leguminosarum strain T83K3 to R. trifolii strain T37. Step 2, recombination event between pJB5JI and pRtT37a resulting in the four classes of transconjugants. I, Class I transconjugants in which R. trifolii strain T37 nif and nod genes (hatched boxes) have been deleted; R. leguminosarum strain T83K3 nod and nif genes (open boxes) are present. II, Class II transconjugants in which strain T83K3 nif genes have been deleted; strain T83K3 nod and R. trifolii nif and nod genes are present. III, Class III transconjugants which contain both sets of nif and nod genes; DNA from one or both plasmids may be deleted. IV, Class IV transconjugants which contain two "hybrid" plasmids. Tn 5 is located on a different plasmid from the nif genes. The location of the R. leguminosarum nod genes is not definite.


Tn5
pJB5JI
nif
nod
pRtT37a
nod
nif
R. leguminosarum strain T83K3
R. trifolii strain T37
step 1
Tn5
nif
nod
nod
step 2
Tn5
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CHAPTER III

Characterization of a Spontaneous Non-nodulating Mutant of *Rhizobium trifolii* Strain 0403: Linkage of Nodulation and Nitrogen Fixation Genes.

INTRODUCTION

Bacteria of the genus Rhizobium are capable of forming a species-specific symbiosis with leguminous plants. The presence of large plasmids (>100 Mdal) appears to be a general feature of strains of Rhizobium (35,72,137,146). Several reports have shown that genes required for nodulation (nod) of legumes and for nitrogen fixation (nif) are plasmid-encoded (5,11,79,94,100,123,147,164). Historically, many investigators have reported instability of nodulation and nitrogen fixation phenotypes in strains of Rhizobium following long term storage or growth on certain media (2,58,117,139,154,175). More recently, non-nodulating (Nod^-) isolates of R. leguminosarum and R. meliloti have been obtained which contain deletions of plasmid DNA (5,11,164).

Results presented in this chapter describe the isolation and characterization of a mutant of R. trifolii strain 0403 which contains a large deletion of DNA in the smallest plasmid. Evidence presented herein indicated that the nif structural genes and gene(s) essential for nodulation are located on this region of deleted plasmid DNA.

MATERIALS AND METHODS

Materials. All of the materials used in this chapter have been described in Chapters I and II.

Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this chapter have been described in Chapter II. Plasmid-deletion mutants of R. trifolii strain 0403 were detected in a culture of strain 0403 stored for about one year at 4°C on a BMM medium (7) agar slant. The culture was restreaked on BMM agar plates and 40 isolates were picked. These isolates were numbered sequentially starting with 2001.

Media. In addition to the media described in Chapters I and II, BMM medium (7) was used to culture R. trifolii strains. BMM medium consisted of: 10.0 g mannitol, 0.23 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 1.1 g sodium glutamate, and 1.0 ml each of vitamin and trace element stock solutions per liter of redistilled water (pH 6.8). The trace element stock solution contained: 0.5 g $CaCl_2$, 14.5 mg H_3BO_3, 12.5 mg $FeSO_4 \cdot 7H_2O$, 7.0 mg $CoSO_4 \cdot 7H_2O$, 0.5 mg $CuSO_4 \cdot 7H_2O$, 0.5 mg $MnCl_2 \cdot 4H_2O$, 11 mg $ZnSO_4 \cdot 7H_2O$, 12.5 mg Na_2MoO_4 per 100 ml redistilled water. The vitamin stock solution contained (per 100 ml redistilled water): 12.0 mg myo-inositol and 2 mg each of riboflavin, p-aminobenzoic acid, nicotinic acid, biotin, thiamine·HCl, pyridoxine·HCl, and calcium pantothenate.

Methods. The characterization of the plasmid deletion mutant of R. trifolii strain 0403 was performed using the techniques described in Chapter II.

RESULTS

Bacteria from a culture of R. trifolii strain 0403 stored at 4°C for about one year were observed to have an altered plasmid profile (data not shown). In addition to the four indigenous plasmids of R. trifolii strain 0403, a smaller plasmid was present in the bacteria from this culture. The intensity of fluorescence associated with this plasmid and with pRt0403a, the smallest plasmid of wild-type strain 0403, was less than that observed for the other three plasmids. This culture was restreaked and forty single colony isolates were obtained.

Plasmid Analysis. Plasmid profiles of these isolates were examined using the Ekhardt agarose gel technique (Figure 34). Nineteen isolates were found to have the same plasmid profile as wild-type strain 0403 (Figure 34, lane A). The plasmid profiles of the other twenty-one isolates were altered. The three high molecular weight plasmids, pRt0403b, c, and d, had the same electrophoretic mobility in agarose gels as the corresponding plasmids of wild-type strain 0403. The smallest plasmid in these twenty-one isolates migrated faster than pRt0403a. Apparently, a large segment of DNA had been deleted from pRt0403a to generate the new, smaller plasmid.

To determine the approximate size of the deletion, the molecular weights of pRt0403a and the plasmid-deletion derivative, termed pRt0403aΔ, were estimated. A standard curve was constructed using plasmids of known molecular weight as described in Chapter II (Figure

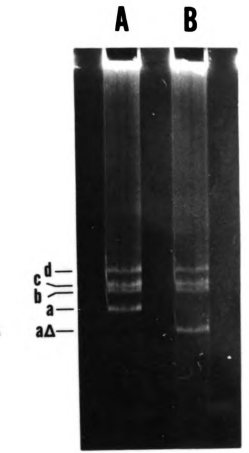


Figure 34. Ekhardt agarose gel electrophoresis of R. trifolii strain 0403 and strain 2016. Lane A, strain 0403; B, strain 2016 containing pRt0403aΔ. Abbreviations of plasmid designations are indicated on the left.

35). The molecular weight of pRt0403a was estimated to be 290 x 10^6. The molecular weights of the other three plasmids of R. trifolii strain 0403 were estimated by extrapolation of the curve above 325 x 10^6. The estimated molecular weights were 335 x 10^6, 350 x 10^6 and 390 x 10^6 for pRt0403b, c, and d, respectively.

Unfortunately, the log relative mobility of pRt0403aΔ is located on the non-linear portion of the curve. Since the shape of the curve through this region is not known, only an approximate molecular weight can be obtained. By extrapolation of both linear segments of the curve, upper and lower estimates of the molecular weight of pRt0403aΔ of 250 x 10^6 and 185 x 10^6, respectively, were obtained. This gives an average molecular weight of about 220 x 10^6, indicating that approximately 70 Mdal of DNA had been deleted from pRt0403a.

Clover Nodulation Test. The symbiotic properties of the isolates from the mixed culture of strain 0403 were examined on the host plant, white clover (Table 11). The twenty-one isolates harboring pRt0403aΔ did not nodulate clover plants, whereas, all eighteen isolates possessing the wild-type plasmid profile formed nitrogen-fixing nodules (Nod^+Fix^+) on clover roots. Thus, the deleted region of plasmid DNA apparently codes for gene(s) required for nodulation ability.

Fahraeus Slide Analysis of Infection Process. To further characterize the plasmid deletion mutation, the infection process of wild-type strain 0403 and a plasmid-deletion mutant (strain 2016) was examined microscopically using the Fahraeus slide technique.

Bacteria of both strain 2016 and strain 0403 were observed to bind to the tips of clover root hairs (Figure 36, panels a and b). Attachment of Rhizobium to root hair tips has been described as one of the

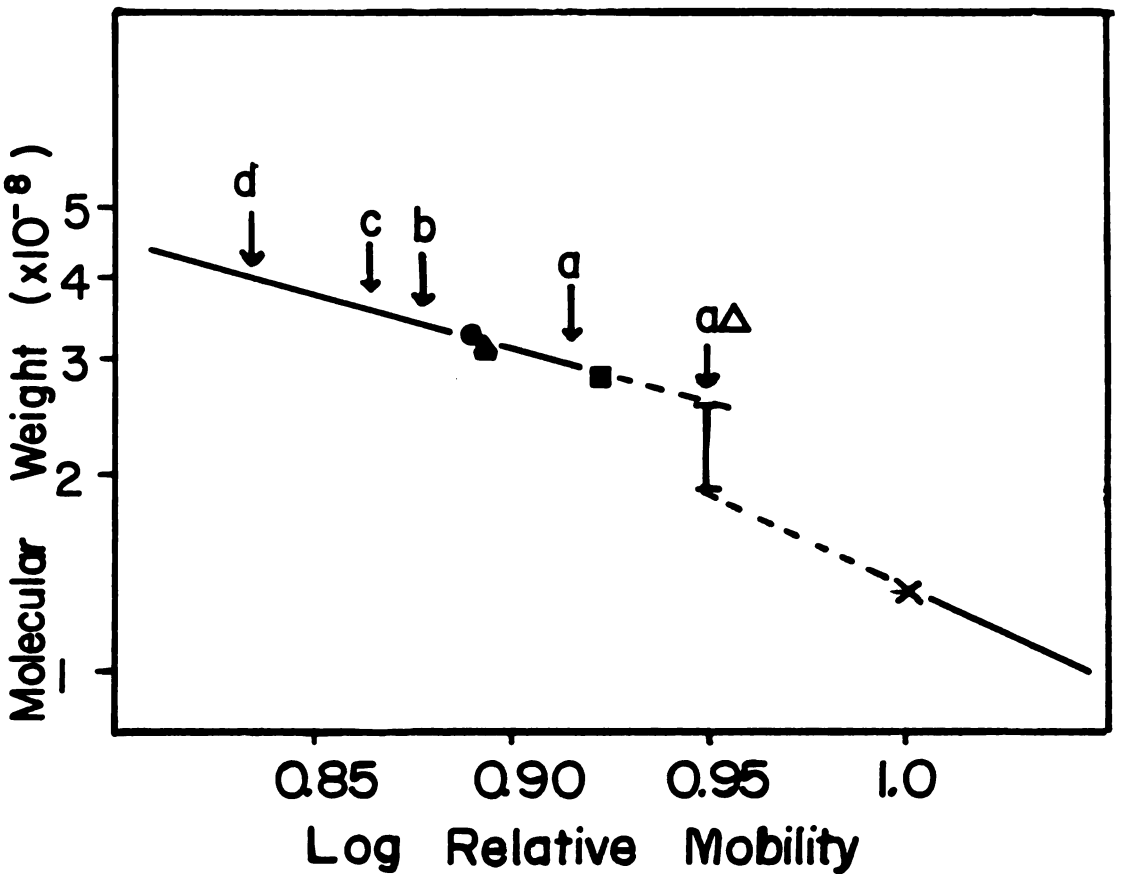


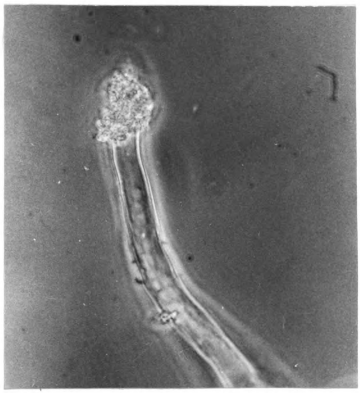
Figure 35. Molecular weight of plasmid DNA versus log relative mobility of plasmids in agarose gels. X, pTiC58, ■, pMG5; ▲, pMG1; ●, pAtC58. The log relative mobilities of the four indigenous plasmids of R. trifolii strain 0403 (a, b, c, and d) and the deletion derivative of pRt0403a (denoted aΔ) are indicated by the arrows. The line drawn through pTiC58 (X) was constructed as described in Figure 15.

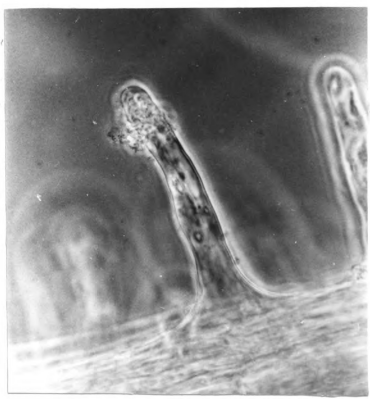
Table 11. Comparison of the symbiotic properties of isolates of R. trifolii strain 0403 containing pRt0403a or pRt0403aΔ.

Plasmid Present in Isolate	Number Tested	Number Nod^+Fix^+
pRt0403a	18	18
pRt0403aΔ	21	0

White clover seedlings were grown aseptically on Jensen agar slants in enclosed tubes. Plants were inoculated with the respective culture and after five weeks growth, scored for nodules (Nod^+) and tested for acetylene reduction ability (Fix^+).

Figure 36. Attachment of R. trifolii to clover root hair tips. Panel a, strain 0403 containing pRt0403a; panel b, mutant strain 2016 containing pRt0403aΔ.



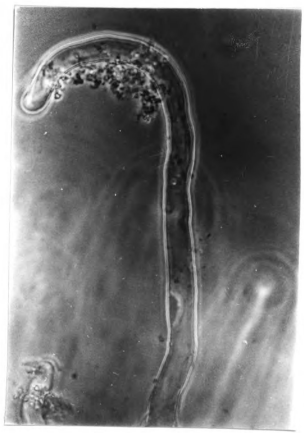


initial steps in the infection process (6,43,44,48). Curling of root hairs of clover plants inoculated with either strain 2016 or strain 0403 was also observed (Figure 37, panels a and b). Infection threads were induced in very tightly curled root hairs of plants inoculated with R. trifolii strain 0403 (Figure 38). However, neither tightly curled root hairs nor infection threads were observed in plants inoculated with strain 2016. Presumably, DNA coding for attachment of bacteria to clover root hair tips and for the "curling inducer(s)" were not deleted from pRt0403a or are encoded on another region of the genome. A gene(s) coding for an essential process in the development of infection threads was apparently located on the DNA deleted from pRt0403a.

Nif Hybridization Analysis. To determine which plasmid in R. trifolii strain 0403 and strain 2016 codes for the nif genes, ^{32}P-labeled R. meliloti nif DNA was hybridized to a Southern filter of an Ekhardt agarose gel of plasmid DNA from R. trifolii strain 0403 and strain 2016 (Figure 39). The nif probe was observed to hybridize only to pRt0403a in strain 0403. The probe did not hybridize to pRt0403aΔ, or to the other plasmids in strain 2016, indicating that the nif structural genes were probably deleted.

To confirm that the nif genes were indeed deleted from strain 2016 and had not recombined into the chromosome, total DNA from strain 0403 and strain 2016 was isolated, digested with restriction endonuclease Eco RI and examined for the presence of the nif structural genes by Southern blot analysis using K. pneumoniae nif DNA as a heterologous hybridization probe. The ^{32}P-labeled K. pneumoniae nif probe hybridized to a 5.0 kb Eco RI restriction fragment of R. trifolii

Figure 37. Root hair curling of clover plants inoculated with R. trifolii strains. Panel a, strain 0403 containing pRt0403a; panel b, mutant strain 2016 containing pRt0403aΔ.



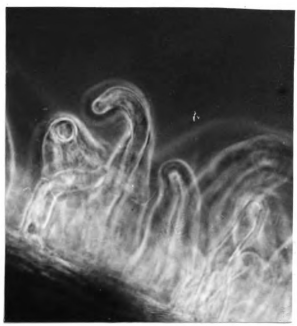
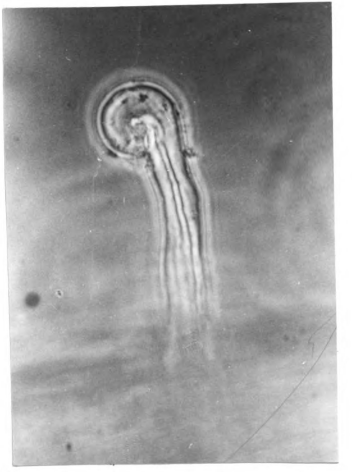


Figure 38. Infection thread in a curled root hair of a clover plant inoculated with R. trifolii strain 0403. The arrow indicates the infection thread. Infection threads were not observed in plants inoculated with R. trifolii strain 2016.



strain 0403 DNA. The probe did not hybridize to any fragments of strain 2016 DNA. Thus, the 5.0 kb Eco RI restriction fragment containing the R. trifolii nif structural genes which is normally present on pRt0403a has been deleted in strain 2016.

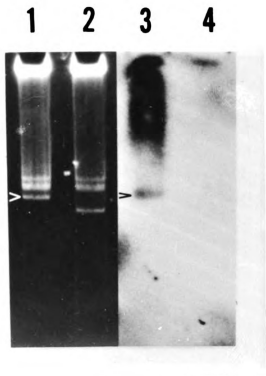


Figure 39. Hybridization of a heterologous nif probe to plasmid DNA from R. trifolii strain 0403 and strain 2016 separated on an Ekhardt agarose gel. Lanes 1 and 2 show the plasmids present in strains 0403 and 2016, respectively. Lanes 3 and 4 show autoradiographs of Southern blots of the plasmid DNA from lanes 1 and 2, respectively, hybridized with ^{32}P-labeled pRmR2. The symbol, >, denotes pRt0403a.

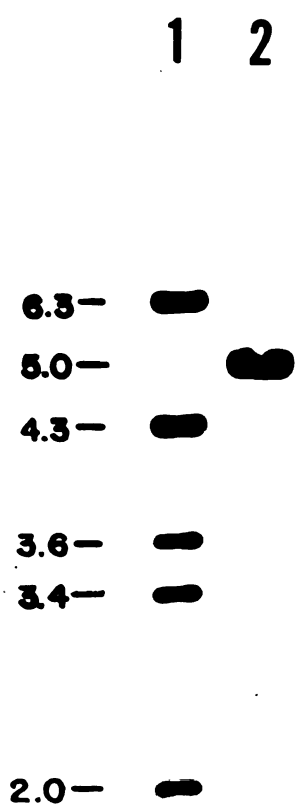


Figure 40. Autoradiogram of a heterologous nif probe hybridized to a Southern blot of Eco RI-digested total DNA of R. trifolii. Lane 1, a mixture of 25 ng of Eco RI digested, 25 ng Eco RI+Bam HI-digested and 25 ng of Eco RI + Hind III-digested pSA30 DNA. Lanes 2 and 3, Eco RI-digested total DNA (5 µg) from R. trifolii strain 0403 and strain 2016, respectively. The heterologous nif probe was the 3.4 k Eco RI-Hind III Fragment of pSA30 (see Figure 14).

DISCUSSION

Four large plasmids are present in R. trifolii strain 0403. Long term storage of this strain resulted in a deletion of about 65 Mdal of DNA from the smallest resident plasmid, pRt0403a. Isolates harboring the approximately 220 Mdal deletion derivative of pRt0403a were unable to nodulate clover plants.

Analysis of the infection process of strain 0403 and strain 2016 by the Fahraeus slide techique, showed that cells from both strains would bind to the tips of clover root hairs and would induce curling and deformation of the root hairs. Strain 2016 could not induce the formation of infection threads. Thus, DNA coding for genes essential for attachment of the bacterial cells to clover roots, and for the curling inducer(s) were either not deleted from pRt0403a or were located on another region of the genome. In contrast, DNA coding for gene(s) essential for the formation of infection threads were deleted from pRt0403a.

Hybridization of a heterlogous nif probe to plasmid DNA from strain 0403 and strain 2016 showed that the nif structural genes, normally present on pRt0403a, had also been deleted. Thus, at least some nif and nod genes are linked and are present on the approximately 70 Mdal region deleted from pRt0403a.

Deletion of DNA coding for genes required for the symbiosis between Rhizobium and legumes have been reported for other species of

Rhizobium (5,11,164). Beringer *et al.* (11) isolated a Nod$^-$ strain of *R. leguminosarum* following UV-mutagenesis. The mutant phenotype was found to be the result of a large deletion in one of the plasmids. Complementation tests with pR11JI, a transmissible plasmid coding for both *nod* and *nif* genes, and symbiotically defective derivatives (Nod$^-$ or Fix$^-$) of pR11JI demonstrated that both *nif* and *nod* genes were located in the deleted region of DNA (22,29). Spontaneous and heat-induced deletions in the "megaplasmid" of several strains of *R. meliloti* have been reported (5,164). These deletions were variable in size and were also found to span both the *nif* strucutral genes and the *nod* genes.

It is interesting to note that most of the observed plasmid deletions have occurred in the region of the *nod* and *nif* genes. Few deletions which produce auxotrophs (i.e., chromosomal) or which occur in plasmid DNA not coding for the *nod* and *nif* genes have been observed. For the latter, this may be due to the lack of genetic markers other than *nif* and *nod*.

The relatively high frequency of loss of the *nif* and *nod* genes suggests there may be a "hot spot" for deletion formation in the region of these symbiotic genes (5). Insertion sequences (IS elements) have been shown to mediate the deletion of variable, but not random, amounts of DNA adjacent to the IS element (33,155). The frequency of deletion formation mediated by the insertion element IS1 can be 100- to 1000-fold above the background rate of spontaneous deletions (155). Both the location and orientation of the IS element affect the frequency of deletions (33,155). The mechanism of deletion formation appears to be closely related to the replication/transposition process (33).

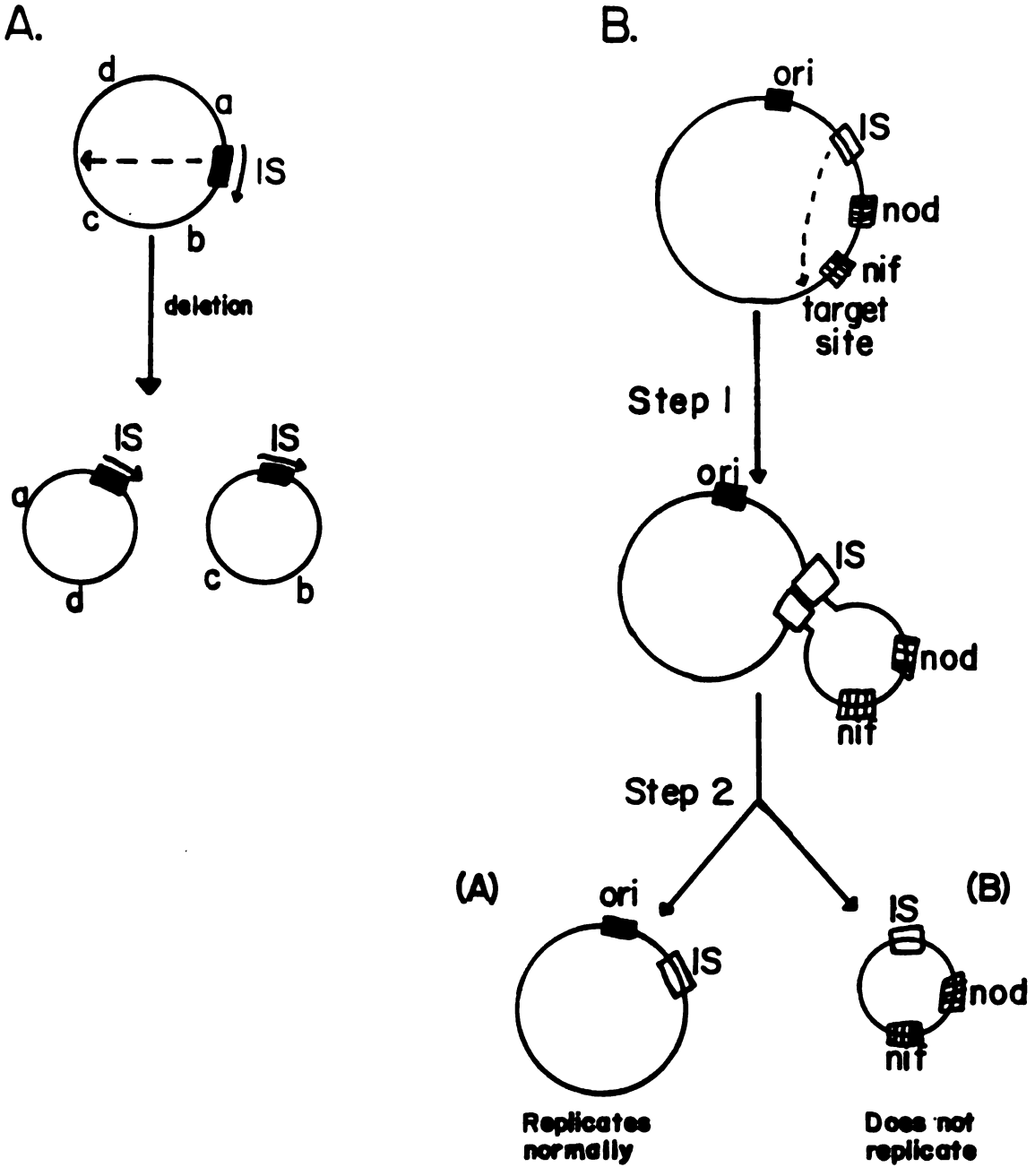
Insertion elements indigenous to Rhizobium have been identified in R. lupini (149) and R. meliloti (167). The R. meliloti insertion element (ISRm1) is a multicopy element which transposes at high frequency into R. meliloti nif DNA. The occurrence of deletions of nod and nif genes in the strain containing ISRm1 was not reported.

While the mechanism for the deletion of nif and nod genes in Rhizobium is at present unknown, an explicitly speculative model for IS-mediated deletion of plasmid DNA containing the nif and nod genes can be postulated (Figure 41, panel B). This model is based on a model (Figure 41, panel A) proposed for deletion formation during transposition of IS elements and transposons (33,181). The insertion element transposes to another site on the plasmid (Figure 41, step 1). During the cleavage, replication, and ligation events necessary for production of the new IS element, two circular DNA molecules are generated (Figure 41, step 2). However, with the orientation shown for the origin of replication (ori), the IS element, the symbiotic genes, and the target site, the circular DNA molecule containing the nif and nod genes (B) would not contain an origin of replication and would be diluted out of the culture by cell division. The other plasmid molecule (A) from which the symbiotic genes have been deleted contains an origin of replication and would be stably maintained. The variation in the size of the deletion would be dependent upon the location of the target site of transposition of the IS element.

To date, IS elements have not been detected in R. trifolii strain 0403, or in any other R. trifolii strains. Until IS elements are demonstrated in R. trifolii strain 0403, the speculative model

presented for the mechanism of deletion of symbiotic genes from pRt0403a cannot be verified.

Figure 41. A speculative model for IS-mediated deletion of plasmid DNA containing nif and nod genes in Rhizobium. Panel A, model proposed by Shapiro (181) for the generation of deletions by insertion elements. Panel B, model for deletion of plasmid DNA encoding nif and nod genes in Rhizobium. Step 1, transposition of insertion element to target site. Step 2, replication of the insertion element at the target site resulting in the production of two plasmid DNA molecules, (A) and (B). Plasmid (A) contains the origin of replication (ori) and will replicate normally. Plasmid B, which contains the nif and nod genes, lacks an ori site and does not replicate. Plasmid (B) will be diluted out of the bacterial population by cell division and plasmid segregation.


A.
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a
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deletion
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ori
IS
nod
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Step 2
(A)
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(B)
IS
nod
nif
Replicates
normally
Does not
replicate

SUMMARY

Large plasmids coding for genes essential for the *R. trifolii* - clover symbiosis have been identified in two strains of *R. trifolii*. The smallest of the three plasmids of *R. trifolii* strain T37 and of the four plasmids of *R. trifolii* strain 0403 were shown to code for the nitrogenase (*nif*) structural and nodulation (*nod*) genes. A non-nodulating mutant of strain 0403 containing a spontaneous deletion of about 70 Mdal of plasmid pRt0403a DNA was isolated. This mutant was able to attach to and induce the curling of root hairs but was unable to induce the formation of infection threads. Hybridization analysis using a heterologous *nif* probe indicated that the *nif* structural genes, which are encoded on pRt0403a, had been deleted. Thus the *nif* genes and genes essential for nodulation are encoded within a 70 Mdal region of pRt0403a.

Transfer of pJB5JI, a *R. leguminosarum* plasmid coding for pea nodulation and *nif* genes, into *R. trifolii* strain T37 generated transconjugants containing a variety of plasmid profiles. The altered plasmid profiles resulted from the recombination of pJB5JI with the smallest plasmid, pRtT37a, of strain T37. The plasmid profiles of the transconjugants and the symbiotic properties exhibited on both peas and clover were stably maintained even after reisolation of the transconjugants from root nodules. The 140 transconjugants were grouped into four classes based on the plasmid profile and the symbiotic properties

exhibited on both host plants.

Class I transconjugants were unable to nodulate clover but formed effective nodules on peas. The R. leguminosarum nif genes were present but the R. trifolii nif genes had been deleted. These strains contained a plasmid of molecular weight corresponding to pJB5JI. Southern analysis of total DNA using pJB5JI as a hybridization probe indicated that the Class I strains contained less pJB5JI sequences than the pJB5JI donor, R. leguminosarum strain T83K3. Similar analysis with pRtT37a indicated that some pRtT37a sequences were also present.

The Class II transconjugant strains formed nodules on both host plants. However, N_2-fixing nodules were only formed on pea plants. Hybridization analysis indicated that the R. leguminosarum nif genes had been deleted. Although the R. trifolii nif genes were present, these genes could not complement the deleted R. leguminosarum nif genes in pea nodules, suggesting that the regulation of R. leguminosarum nif genes may be different than that for the nif genes in R. trifolii. Strains of Classes III and IV formed effective nodules on both host plants. Both sets of symbiotic genes were present in these strains and were functional. Class III strains contained a single "hybrid" plasmid larger then pRtT37a, while Class IV strains contained two plasmids of molecular weights intermediate to the molecular weights of pJB5JI and pRtT37a.

Several unique findings were observed during this research. Transfer of pJB5JI to R. trifolii strain T37 resulted in recombination between pJB5JI and pRtT37a, both of which encode genes essential for the Rhizobium - legume symbiosis. Approximately 30% of the transconjugants (Class I) were stably converted to a R. leguminosarum strain.

Other transconjugants possessed a host range extended to include pea plants, a first step toward extension of the host range of Rhizobium to include non-leguminous plants.

Several of the strains isolated during this research may be useful for further research on the symbiosis. The R. trifolii strain 0403 non-nodulating mutant containing a large deletion of plasmid DNA could be utilized in the study of genes required for the symbiosis by complementation analysis. The Class II transconjugants may be beneficial in the study of the regulation of nif expression in pea nodules.

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