PLASMIDS IN *Rhizobium Phaseoli*

A THESIS PRESENTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE IN MICROBIOLOGY
AT MASSEY UNIVERSITY

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1982

ABSTRACT

Fast growing strains of *Rhizobium* have been divided into four homology groups on the basis of DNA hybridization. Rhizobia from two of these homology groups can form effective nodules with beans (*Phaseolus vulgaris*). Large plasmids associated with nodulation have been demonstrated in *Rhizobium* sp. A study was undertaken to examine the plasmids in rhizobia from the two different homology groups capable of nodulating beans.

The effectiveness of strains of *Rhizobium phaseoli* on bean plants were examined. Spontaneous antibiotic resistant mutants which retained the ability to nodulate beans were selected. Antibiotic resistance marked clones were incubated at elevated temperatures to produce an ineffective mutant stain of *Rhizobium phaseoli* NZP 5492.

Methods of extracting large plasmids from *Rhizobium phaseoli* were developed. Plasmids were visualised by agarose gel electrophoresis and purified by cesium chloride - ethidium bromide density gradient ultracentrifugation.

We demonstrated the presence of plasmids of molecular weight range 66 Md to 316 Md in *Rhizobium phaseoli* strains. Some strains contained a single plasmid while others contained multiple plasmids.

Comparisons were made between whole plasmids and restriction endonuclease digests of the plasmids from the two groups. The fragment pattern obtained from *Eco* RI digests showed differences in fragment numbers and size between plasmids from DNA homology group 1 and DNA homology group 2.

Further studies using gel blotting and hybridization techniques are required to ascertain the degree of homology between the plasmids, both within groups and between groups.

Rhizobium phaseoli NZP 5479 and NZP 5547 a non-nodulating mutant of strain NZP 5479 were examined. Both strains had plasmids of estimated molecular weights 186 Md and 288 Md. There was no detectable difference in the size of the plasmids in the non-nodulating mutant compared to the effective parent strain.

Rhizobium phaseoli NZP 5492 B5/8 (effective) and NZP 5492 B5/1 an ineffective mutant obtained from strain NZP 5492 had plasmids of similar molecular weight. Differences were observed in the *Eco* RI fragment pattern and possible rearrangements to the DNA to account for these differences are presented.

ACKNOWLEDGEMENTS

I am indebted to the Department of Microbiology and Genetics, Massey University, for providing the opportunity and facilities for this investigation.

In particular I would like to thank my supervisor, Dr B.D.W. Jarvis for his encouragement and guidance.

I would also like to thank:

Professor D.F. Bacon and the academic and technical staff of the Department of Microbiology and Genetics for general help and interest;

Dr D.B. Scott (D.S.I.R.) for strains NZP 5479 and NZP 5547, the use of glasshouse facilities, assistance with the Anderson plasmid extraction method and general discussions;

Miss C.P. Liddane (D.S.I.R.) for assistance with plant nodulation testing;

Dr J.G. Robertson (D.S.I.R.) for supply of pumice;

Dr A.W. Jarvis (D.R.I.) for gifts of phage λ;

Dr A.J. Robinson (Vet, Pathology and Public Health) for gifts of *Eco* RI, phage λ, use of the ultracentrifuge and for informative discussions;

Mr J. Clouston and the staff of the Central Photographic Unit for assistance with photography;

Mr H.F. Neilson (Horticulture and Plant Health) for assistance with the nodule photographs;

Mrs J. Trow for the diagrams;

Mrs V. Fieldsend for the typing.

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INTRODUCTION

The process of nitrogen fixation by microorganisms is of worldwide importance. In New Zealand agriculture the most important aspect of biological nitrogen fixation is the symbiotic association between the genus *Rhizobium* and the roots of white clover plants. Mackinnon *et al* (1975) estimated that 97% of the nitrogen used in New Zealand is fixed biologically. It is clear that the agricultural economy of New Zealand depends largely on the process of biological nitrogen fixation. In the absence of this process the increased cost of producing extra nitrogenous fertilizers would be prohibitive. Comparatively minor increases in the efficiency of the *Rhizobium* - legume symbiosis could considerably increase the productive capacity of New Zealand agriculture.

The genetics of *Rhizobium* and the genetic basis of the symbiotic association with legumes are the subjects of much research. Brill (1977) has speculated that if the nitrogen-fixing activities of bacteria can be understood, then the improvements which might be made could result in a reduced dependence on nitrogenous fertilizer.

The eighth edition of Bergey's Manual of Determinative Bacteriology classifies the genus *Rhizobium* on the basis of cross inoculation groups. This classification is inadequate mainly due to the considerable overlap which exists between cross inoculation groups. The isolation of bacteria which nodulate plants in more than one cross inoculation group is common place (Wilson 1944, Graham 1964) and many of the slow growing *Rhizobium* strains have a very wide range of legume hosts and do not fall readily into a cross inoculation grouping.

Other methods of classifying the genus which have been proposed by *Rhizobium* taxonomists include numerical taxonomy (Graham 1964, t'Mannetje 1967, White 1972), phage typing (Staniewski 1968) and DNA hybridization (De Ley *et al* 1965, Gibbins and Gregory 1972).

Jarvis *et al* (1980) determined DNA homologies among 27 strains of *R. trifolii*, 4 strains of *R. leguminosarum* and 4 strains of *R. phaseoli*. They proposed that *R. trifolii* and *R. leguminosarum* be combined under the species name *R. leguminosarum*. They found that the average relatedness of *Rhizobium* strains from *Phaseolus vulgaris* to those obtained from clover was 46% and concluded that *R. phaseoli* should be retained as a separate species until it was examined in more detail.

Crow *et al* (1981) studied the DNA homology between 113 strains of fast growing acid producing rhizobia and seven reference *Rhizobium* strains. They divided these strains into four homology groups: Group 1 comprised *R. trifolii*, *R. leguminosarum* and *R. phaseoli*, all consolidated under the name *R. leguminosarum*; Group 2 comprised the *Rhizobium* strains from *Coronilla* sp. and some strains from *Sophora* and *Onobrychis* sp.; Group 3 comprised *R. meliloti* and group 4 comprised strains from a variety of hosts including *Lotus corniculatus*, *Lotus tenuis* and *Lupinus densiflorus*. DNA homology Group 1 includes *Rhizboium phaseoli* strains, that is those isolated from *Phaseolus vulgaris* and also strains isolated from *Neptunia gracilis* which can form effective nodules on *Phaseolus* as well as strains from *Pisum* and *Vicia* which form ineffective nodules on *Phaseolus*. DNA homology group 2 strains can form effective nodules on *Phaseolus vulgaris* but have low (18-30%) homology with all reference strains from group 1.

Plasmids are circular self-replicating molecules of extra-chromosomal DNA found in many species of bacteria. The family Rhizobiaceae contains the genera *Agrobacterium* and *Rhizobium* (Jordan & Allen, 1974). All species of *Agrobacterium* contain large plasmids which are associated with the virulence of the species (Van Larebeke *et al*1974, Zaenen *et al* 1974).

Loper & Kado (1979) and Thomashow *et al* (1980) have shown that the host range of *Agrobacterium tumefaciens* is primarily determined by the Ti plasmid. Thomashow *et al* (1981) studied the relationship between the limited host range octopine Ti plasmids and the wide host range octopine Ti plasmids and found only 6-15% homology between the two groups leading to the conclusion that two distinct families of plasmids were involved in specifying host range.

Symbiotic properties such as nodule formation and nitrogen fixation are known to be unstable in some *Rhizobium* isolates. Evidence of plasmid involvement in symbiosis and host range specificity was first reported by Higashi (1967) who transfered the ability to nodulate clover from a *R. trifolii* strain to a *R. phaseoli* strain. Higashi also found a loss of infectiveness in both *R. trifolii* and *R. phaseoli* following treatment with acridine orange. Since acridine dyes are known plasmid eliminating agents in gram negative bacteria, these results were further evidence for the involvement of plasmids in nodulation. Dunican and Cannon (1971) demonstrated a loss of effectiveness in *R. trifolii* strains following treatment with known plasmid eliminating agents.

Other evidence for plasmids in *Rhizobium* includes that of Hirsch (1979) who reported on the transfer of bacteriocins between *R. leguminosarum* strains, Johnston *et al* (1978) transferred the ability to nodulate peas to *R. trifolii* , *R. phaseoli* and an ineffective strain of

R. leguminosarum . Brewin *et al* (1980) showed that the particular host range characteristics of a *R. leguminosarum* strain were due to plasmid borne information. They were able to transfer this information to another *R. leguminosarum* strain changing the host range of the second strain. Buchanan-Wollaston *et al* (1980) demonstrated both Fix^+ & Nod^+ characteristics on plasmids in *R. leguminosarum*. . Scott and Ronson (1982) demonstrated the presence of a nodulation plasmid in *R. trifolii* and showed the transfer of nodulation ability from Nod^+ Fix^+ strains of *R. trifolii* to Nod^- plasmid cured derivatives.

The physical presence of plasmids in Rhizobium was suggested by DNA density studies (Sutton 1974) and plasmid DNA was demonstrated in some strains by dye-bouyant density ultracentrifugation (Tshitenge *et al* 1975, Zurkowski and Lorkiewicz 1976). Plasmids of molecular weights ranging from 25-60 mega daltons were reported. At the same time large plasmids of molecular weight 100-160 Md were demonstrated in the other genus of the *Rhizobiaceae* , *Agrobacterium* (Van Larebeke *et al* 1974) Nuti *et al* (1977) applied the techniques developed for the extraction of large plasmids in *Agrobacterium* to *Rhizobium* sp. and demonstrated the presence of large plasmids of molecular weight up to 400 Md . The presence of a large plasmid of greater than 300 Md has been demonstrated as a general feature of *R. meliloti* (Rosenberg *et al* 1981). The nitrogenase genes and some genes controlling an early step in root infection are carried on these plasmids. Large plasmids in *Rhizobium* spp. have also been demonstrated by electron microscope studies (Schwinghamer and Dennis 1979).

Since the demonstration of such large plasmids in rhizobia . much effort has been spent on the development of procedures for the extraction and characterisation of large plasmids. The cleared lysate procedure for the isolation of plasmids (Clewell and Helinski, Guerry *et al* 1973) is very effective with many strains of gram negative bacteria. Cell lysis

with detergent followed by centrifugation allows the separation of plasmid DNA from the pelleted chromosome-membrane complex. This procedure is effective for small plasmids (25-65 Md) in *Rhizbium* but it is unsuitable for large CCC DNA molecules in both *Agrobacterium* and *Rhizobium* . The large plasmids are probably not dissociated from the chromosome-membrane complex during the cleared lysate treatment.

Large plasmids are difficult to isolate because of their association with the chromosomal-membrane complex and the fact that plasmid isolation procedures depend on separation of the intact ccc form of the plasmid molecule. The larger a plasmid is the more sensitive it is to nuclease (enzyme) degredation or mechanical shearing (Denarie *et al* 1981).

The loss of the nod phenotype has been correlated with the loss of plasmids (Prakash *et al* 1978; Zurkowski and Lorkiewicz 1978; Casse *et al* 1979; Hooykaas *et al* 1981) or with the generation of a deletion within a plasmid (Beynon *et al* 1980; Denarie *et al* 1981, Hirsch *et al* 1980, Kondorosi *et al* 1981).

Phaseolus vulgaris is nodulated by genetically distinct groups of rhizobia (Crow *et al* 1981). One group includes strains of *R. phaseoli* isolated from *Phaseolus vulgaris* , the second group contains strains with low homology to group 1 but which can still form effective nodules on *Phaseolus vulgaris* . The involvement of large plasmids in nodulation of *Rhizobium* spp. has been clearly demonstrated. Consequently it was of interest to determine whether strains from different homology groups contained common plasmids.

The aims of this project were:

1. to find a suitable procedure for the isolation of large plasmids from the *R. phaseoli* strains studied.

2. to compare the plasmids of *R. phaseoli* strains derived from different DNA homology groups.

3. To generate nod^- or fix^- mutants of an antibiotic marked *R. phaseoli* strain and compare its plasmid composition with that of the nod^+ fix^+ parent.

MATERIALS AND METHODS

1. BACTERIAL STRAINS AND MAINTENANCE

The bacterial stains used in this investigation are given in Tables I and II. The *Rhizobium* strains were maintained on Yeast Mannitol Agar (YMA) slopes at 4°C and subcultured at 2-6 month intervals. Gram stained smears and cultures on Brian Heart Infusion (BHI) agar were used to monitor culture purity. The *Escherichia coli* strains were grown on BHI and the *Agrobacterium* strain on Tryptone Yeast (TY) medium. In addition most of the strains were lyophilised and stored at 4°C in the Microbiology and Genetics Department Collection.

2. MEDIA

2.1 LURIA BROTH contained (g/litre): Tryptone (Difco), 10.0; Yeast extract (Difco), 5.0; Sodium chloride, 0.5. The pH was adjusted to 7.0. After autoclaving (121°C, 30 mins) 10cm^3 per litre of separately sterilized 20% glucose solution were added.

2.2 YEAST MANNITOL BROTH (YMB) (Vincent, 1970) contained (g/litre): Mannitol, 10.0; Yeast extract (Difco), 0.5; Dipotassium phosphate (K_2HPO_4), 0.5; Magnesium sulphate ($MgSO_4.7H_2O$), 0.2; Sodium chloride, 0.1. The pH was not adjusted.

Yeast Mannitol Agar (YMA) was obtained by adding 15g of agar (Davis) to each litre of this medium.

2.3 BRAIN HEART INFUSION BROTH (BHI) was made by dissolving 37g per litre of Brain Heart Infusate (Difco) in distilled water. For solid medium, 15g of agar (Davis) were added per litre of medium.

TABLE I: BACTERIAL STRAINS CONTAINING PLASMIDS USED AS MOLECULAR WEIGHT REFERENCES

Strain		Plasmid molecular Weight (Md)
Escherichia coli	PB1335	3.8
Escherichia coli	R6K	26
Escherichia coli	R100-1	60
Rhizobium meliloti	U45	101
Agrobacterium tumefaciens	C58(a)	130
Agrobacterium tumefaciens	C58(b)	275

TABLE II: RHIZOBIUM STRAINS INVESTIGATED

Strain	NZP number	Culture+ number	Alternative designations	Plant origin
A. HOMOLOGY GROUP 1#				
Rhizobium phaseoli	5097	266		*Phaseolus vulgaris*
Rhizobium phaseoli	5232	270		*Phaseolus vulgaris*
Rhizobium phaseoli	5260	271		*Phaseolus vulgaris*
Rhizobium phaseoli	5479	-		*Phaseolus vulgaris*
Rhizobium phaseoli	5547	-		non-nodulating mutant of NZP 5479
Rhizobium phaseoli	5492	360	4001	*Phaseolus vulgaris*
Rhizobium phaseoli	5459	273		*Phaseolus vulgaris*
B. HOMOLOGY GROUP 2#				
Rhizobium sp.	5384	102	116A5	*Onobrychis viciifolia*
Rhizobium sp.	5443	356	146A1	*Sophora secundiflora*
Rhizobium sp.	5456	105	31A5	*Coronilla varia*
Rhizobium sp.	5462	323	CC401	*Coronilla varia*
Rhizobium sp.	5463	436	116A14	*Onobrychis viciifolia*

+ number of the strain in the Microbiology and Genetics Department Culture collection

Group classification according to Crow, Jarvis and Greenwood (1981)

2.4 TRYPTONE-YEAST EXTRACT MEDIUM (TY) (Beringer, 1974) contained (g/litre): Tryptone (Difco), 5.0; Yeast extract (Difco), 3.0; Calcium chloride ($CaCl_2.6H_20$), 1.3; Bacteriological Agar (Davis), 15.0.

2.5 ANTIBIOTIC RESISTANCE MEDIA. Antibiotic solutions were prepared as follows:

2.51 RIFAMPICIN (3-[4-Methyl piperazinylimino methyl] rifamycin SV) (Sigma) was prepared by dissolving 0.05g solid in 20cm³ methanol.

2.52 STREPTOMYCIN. A sterile solution was prepared by injecting 5.0cm³ sterile deionised water into a sterile vial containing 1g streptomycin sulphate (Glaxo).

2.53 SPECTINOMYCIN. Spectinomycin hydrochloride (Trobicin, Upjohn Corp.) 0.05g, was dissolved in 2.0cm³ sterile deionised water.

2.54 NALIDIXIC ACID. Nalidixic acid (Sigma) 0.1g was dissolved in 10.0cm³ deionised water and passed through a 0.22µm membrane filter (Millipore)

All antibiotic solutions were checked for sterility by spotting them onto BHI and YMA plates which were incubated for 3 days at 25°C. The sterile antibiotic solutions were added to YMA which had been autoclaved and cooled to approximately 50°C. The final concentration of all antibiotic solutions was 100µg/cm³.

2.6 WATER AGAR PLATES (for germinating seeds) were made by adding 1.0g agar (Davis) to 100cm³ deionised water, autoclaving at 121°C for 20 mins and pouring into deep glass petri dishes.

2.7 HOAGLANDS NUTRIENT SOLUTION (for plant tests) was prepared from the stock solutions described in Table III. The required volume of sterile stock solution (Table IV) was added to 10l of sterile deionised water.

3. BUFFERS *

3.1 TRIS-EDTA BUFFER (TE) Consisted of 0.05M Tris (hydroxy methyl) amino methane and 0.02M Ethylene diamine tetra acetic acid. ($[CH_2.N(CH_2COOH)\ CH_2COONa]_2.2H_2O$), pH 8.0.

A 10 times concentrated stock solution was prepared by dissolving 60.55g Tris (Sigma 7-9®) and 74.45g EDTA (Analar, BDH) in 1l of deionised water and adjusting the pH to 8.0 with hydrochloric acid.

3.2 TRIS-EDTA-SODIUM CHLORIDE (TES) contained 0.05M Tris, 0.005M EDTA and 0.05M NaCl. A 10 times concentrated stock solution was prepared by dissolving 60.55g Tris, 29.25g NaCl and 18.6g EDTA in 1 litre of deionised water and adjusting the pH to 8.0 with hydrochloric acid.

3.3 TRIS-SODIUM CHLORIDE (TS) contained 0.05M Tris and 0.05M NaCl. A 10 times concentrated stock solution was prepared by dissolving 60.55g Tris and 29.25g NaCl in 1l of deionised water and adjusting the pH to 8.0 with hydrochloric acid.

3.4 TRIS-EDTA-SUCROSE (TE SUCROSE) contained 25mM Tris, 2.5mM EDTA and 10% sucrose, pH 8.0.

3.5 ELECTROPHORESIS BUFFERS:

* (M = moles per litre)

TABLE III: HOAGLANDS NUTRIENT SOLUTION: STOCK SOLUTIONS

	Molarity	g/litre
A.		
K_2HPO_4	1M	17.4
KH_2PO_4	1M	122.5
B.		
$Mg\ SO_4.7H_2O$	1M	246.5
NaCl	0.25M	14.6
C.		
KNO_3	1M	101.1
$Ca(NO_3)_2.4H_2O$	1M	236.2
NH_4NO_3	0.4M	16.0
D.		
K_2SO_4	0.5M	87.1
E.		
$CaCl_2.6H_2O$	1M	219.1
F. (Microelement solution)		
$^{+}H_3BO_3$	0.5ppm	2.86
$MnCl_2.4H_2O$	0.5ppm	1.81
$ZnSO_4.H_2O$	0.05ppm	0.22
$CuSO_4.4H_2O$	0.02ppm	0.08
$CoSO_4.7H_2O$	0.02ppm	0.095
$Na_2MoO_4.2H_2O$	0.02ppm	0.054

$^{+}$ heat to dissolve

TABLE IV: HOAGLANDS NUTRIENT SOLUTION

Stock solution	Volume added to 10 litres +N	Volume added to 10 litres -N
A	10 cm^3	10 cm^3
B	20 cm^3	20 cm^3
C	45 cm^3	0 cm^3
D	45 cm^3	45 cm^3
E	45 cm^3	45 cm^3
F	10 cm^3	10 cm^3

3.51 ACETATE (E buffer) contained 40mM Tris, 5mM sodium acetate and 1mM EDTA, pH 7.8. A 10 times concentrated stock solution was prepared by dissolving 96.8g Tris, 13.6g $CH_3COONa.3H_2O$ and 7.4g EDTA in 2 litres of deionised water. The pH was adjusted to 7.8 with glacial acetic acid.

3.52 ACETATE - EDTA (for use with sea plaque gels where EDTA would be inhibitory to subsequent enzyme digests). This buffer was prepared as for E buffer, except EDTA was omitted.

3.53 BORATE electrophoresis buffer contained 89mM Tris, 2.5mM EDTA and 8.9mM boric acid, pH 8.2. A 10 times concentrated stock solution was prepared by dissolving 107.7g Tris, 9.3g EDTA and 5.5g boric acid in 1 litre of deionised water.

3.6 RESTRICTION ENDONUCLEASE BUFFERS:

3.61 *Eco* RI buffer contained 0.2M Tris, 0.1M NaCl and 0.01M $MgCl_2$ adjusted to pH 7.5 with hydrochloric acid.

3.62 5X *Eco* RI buffer contained 0.5M Tris, 0.5M NaCl 0.05M $MgCl_2$ and 0.5mg/cm^3 bovine serum albumin (BSA)

3.63 *Hind* III buffer contained (g/litre): NaCl, 3.51; $MgCl_2$, 1.42; Tris, 0.85. The pH was adjusted to 7.4 with HCl.

3.64 *Bam* HI buffer contained (g/litre): NaCl, 8.78; $MgCl_2$, 1.23; Tris, 0.73. The pH was adjusted to 7.9 with HCl.

4. MATERIALS

4.1 PROTEASE

Either Pronase P (Sigma P5130, Type VI from *Streptomyces griseus*) or Pronase E (Sigma P5147, Type XIV from *Streptomyces griseus*) were made up to 10 mg/cm^3 in TE buffer and self digested by incubating at 37°C for 90 mins, then stored in 10cm^3 aliquots at -20°C.

4.2 DIALYSIS TUBING

Cellulose dialysis tubing (Union Carbide) of appropriate diameter was cut to the required length and boiled in deionised water for 10 mins before use.

4.3 ACID WASHED GLASSWARE

Glass vials, screw capped kimax tubes and other glassware used for DNA storage were acid washed by boiling for 10 mins in a 1:3 mixture of concentrated nitric acid and deionised water. The glassware was then drained and boiled for 10 mins in deionised water, drained and rinsed in fresh deionised water and dried in an oven overnight.

4.4 SOLUTIONS FOR DEVELOPING KODAK TRI-X FILM:

4.41 DEVELOPER. Deionised water (4l) was placed in a 5l flask and brought to 38°C in a waterbath. A can of Kodak D-19 developer (801g) was added with continuous stiring and dissolved. The volume was made up to 5l. Developer solutions were stored in tightly stoppered full bottles at room temperature and discarded when they became noticeably yellow in colour.

4.42 FIXER. Ilford Hypam Rapid Fixer concentrate (300cm^3) and Ilford Hardener (38.2cm^3) were added to 1200cm^3 deionised

distilled water. The working strength fixer which resulted was stored in a screw capped glass bottle.

4.5 REDISTILLATION OF PHENOL:

Phenol was purified by distillation. The glass distillation apparatus shown in Figure 1 was constructed from standard 'Quickfit' apparatus. Solid phenol (1.5kg or an equivalent amount of liquid) was placed in flask B. A small volume of water was added to liquefy solid phenol and aluminium turnings (1.5g, 0.1%) and $NaHCO_3$ (0.8g, 0.05%) were added.

The flask was heated to 100°C, at which stage water was boiled off. After removal of water, the temperature rose and a vacuum of 100mm Hg was applied by opening tap F. At this pressure the mixture boils at 174°C and the phenol condenses as a colourless liquid in tube D, and collects in flask E. The vacuum was adjusted with tap G.

Redistilled phenol was stored frozen at -20°C or saturated with the required buffer.

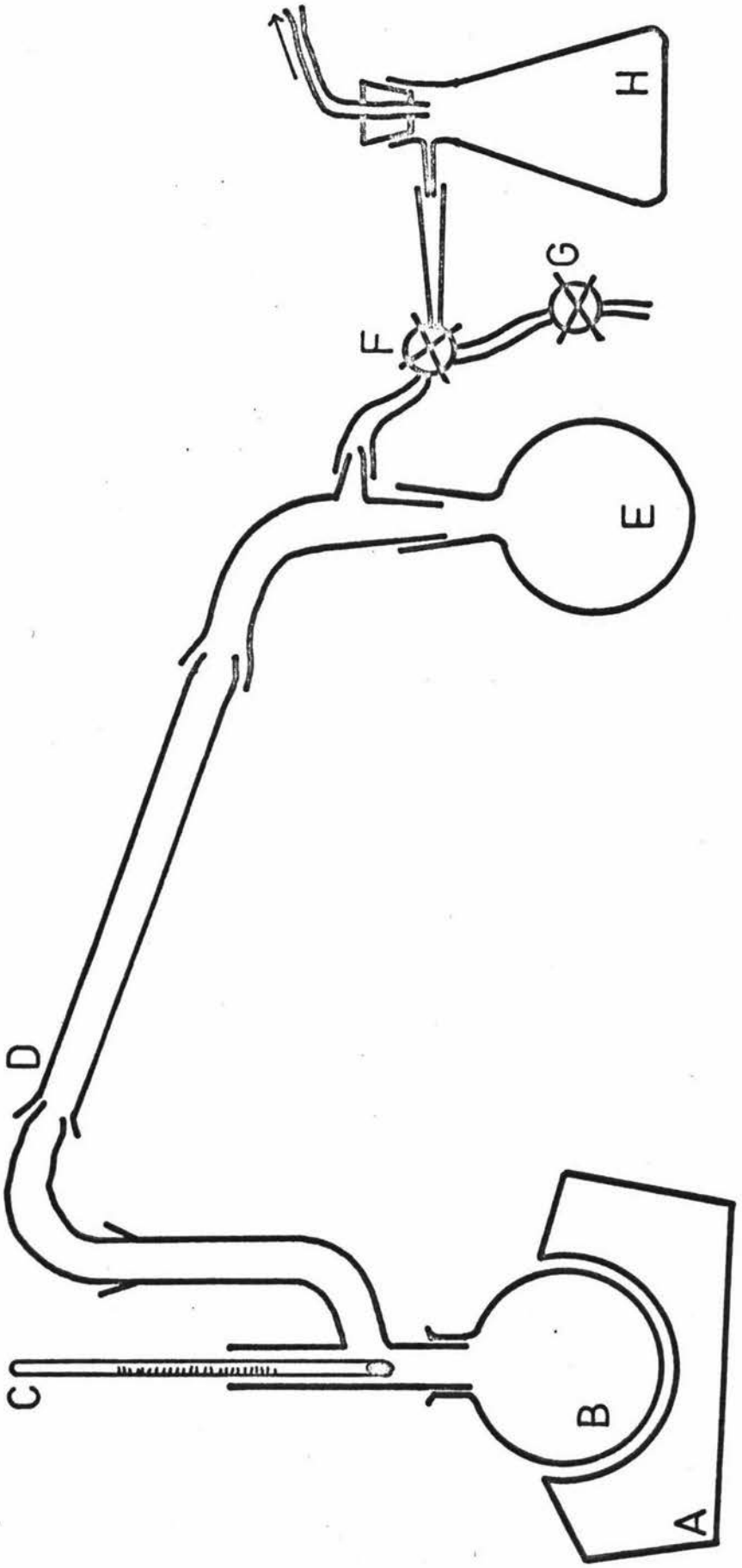
Saturation of phenol was carried out in a 500cm³ separating funnel. 200cm³ of liquid redistilled phenol was shaken with 100cm³ of the required buffer. After standing for the aqueous and phenolic phases to separate, the phenolic (lower) phase was collected into a suitable screw capped glass container and stored frozen at -20°C.

4.6 PRODUCTION AND MAINTENANCE OF CARBON DIOXIDE FREE WATER

Carbon dioxide (CO_2) free water was prepared by boiling about 1.5l of deionised distilled water in a 2l flask and inserting a rubber stopper containing a carbon dioxide trap. The CO_2 trap consisted of a hypodermic needle and a 10cm³ disposable syringe packed with Carbsorb (BDH).

FIGURE 1: Apparatus for the distillation of phenol.

A. Heating mantle
B. 2l Quickfit flask
C. Thermometer
D. Glass tube
E. 1l Quickfit flask
F. Tap
G. Tap
H. Water trap


H
G
F
E
D
C
B
A

4.7 STOCK SOLUTIONS FOR SUCROSE GRADIENT CENTRIFUGATION

4.71 SUCROSE STOCK SOLUTION contained (in phosphate buffer) 66% sucrose; 0.05M $Na_2HPO_4.12H_2O$, 0.025M KH_2PO_4, 0.5M NaCl, 10^{-3}M Mg^{++}, pH 7.2.

Solutions for forming gradients were prepared by diluting this stock solution with deionised distilled water (Table IV). All solutions were stored at 4°C.

4.72 SUCROSE CHASE SOLUTION for gradient fractionation was prepared by dissolving 100g sucrose in 100cm³ deionised distilled water. The solution was stored at 4°C.

5. METHODS

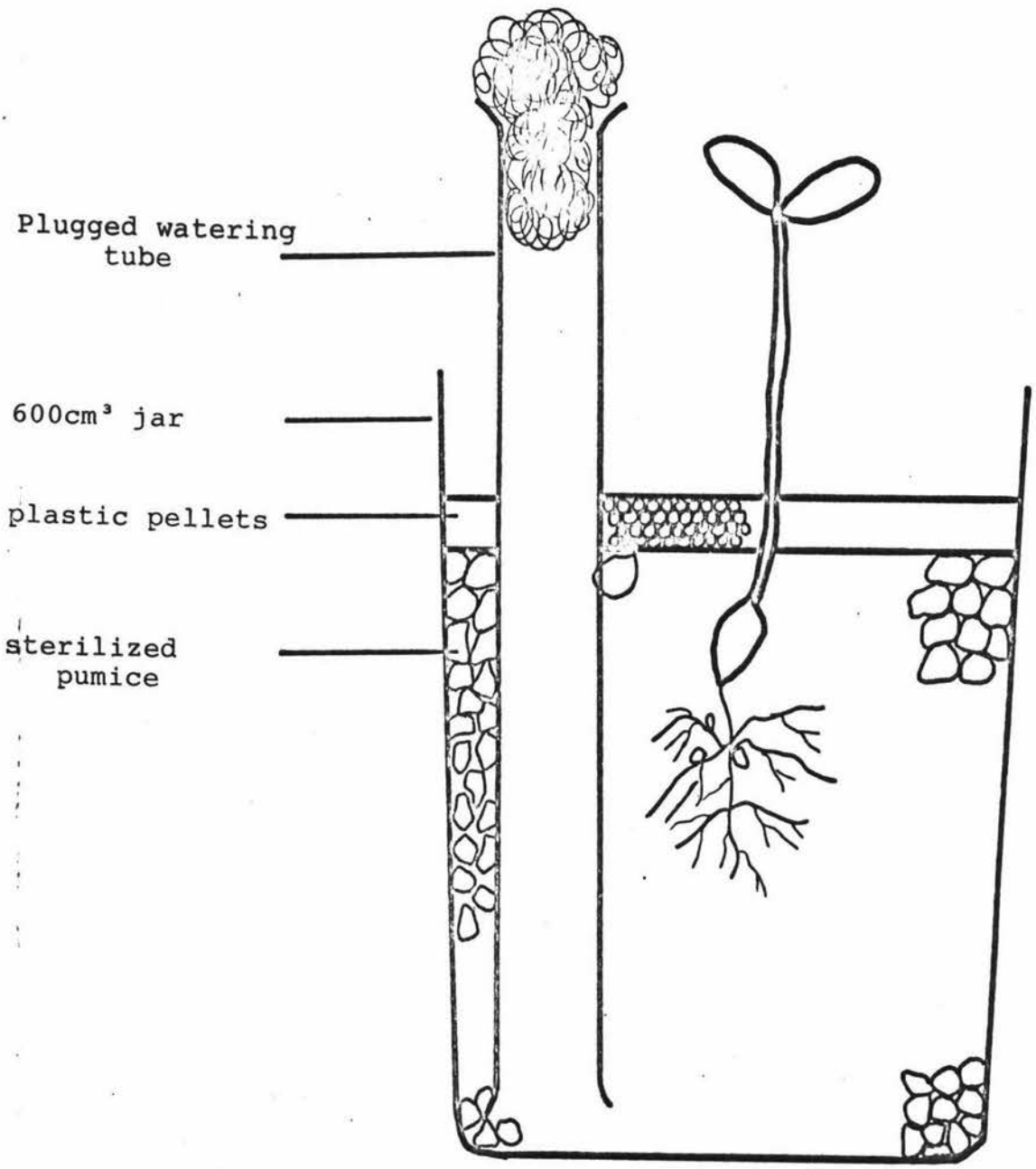
5.1 NODULATION TESTS (Moustafa and Greenwood, 1967)

Seeds were surface sterilized in a stoppered 100cm³ flask by immersion for 5 mins in a mixture containing equal volumes of 30% hydrogen peroxide and 95% ethanol. The seeds were drained and rinsed twice with sterile distilled water, and 10-15 seeds were aseptically placed on water agar plates (Section 2.6). The seeds were germinated in the dark.

Glass jars (600cm³ capacity) containing pumice (grade B) and a glass watering tube (Figure 2) were covered with aluminium foil and autoclaved (1hr at 121°C). Plastic beads were separately sterilised in foil covered containers at 121°C for 20 mins.

The sterile growth system was watered through the watering tube with Hoaglands nutrient solution (Section 2.7). When the seeds had germinated three seeds of approximately the same size and radicle length were selected and aseptically planted in the pumice. The pumice was overlaid with a 1cm

FIGURE 2: Growth system for testing nodulation of bean plants



layer of sterile plastic beads and the jar placed in the dark until the seedlings emerged.

After seedling emergence the jars were transferred to the glasshouse where they were watered as required with sterile water.

After 6-8 weeks the plants were removed from the jars and examined. Nodule characteristics and the weight of the wet and dried tops were recorded.

5.2 REISOLATION FROM NODULES

Nodules were removed and surface sterilized by imersion, for 2 to 5 mins depending on the size of the nodule, in a solution containing equal parts of 95% ethanol and 30% hydrogen peroxide. Sterilized nodules were rinsed 3-4 times in sterile distilled water. The nodules were placed on a flamed microscope slide and squashed with a second microscope slide to release the milky fluid containing the bacteria which was spread on a YMA plate.

5.3 SELECTION OF SPONTANEOUS ANTIBIOTIC RESISTANT MUTANTS

5.31 Method adopted for *R. phaseoli* strain NZP 5492

Yeast Mannitol broth ($50cm^3$) was inoculated and grown into the stationary phase (3 days). $0.5cm^3$ of the culture was plated onto antibiotic media (Section 2.5), BHI and YMA. Sterile toothpicks were used to transfer streptomycin resistant colonies to plates containing BHI, YMA and YMA+ streptomycin, which were marked with numbered grids.

Verified streptomycin resistant colonies were grown in YMB and $0.5cm^3$ was plated onto rifampicin and spectinomycin antibiotic plates. The double resistant mutants obtained were verified by plating on BHI, YMA and antibiotic plates and by gram staining. The effectiveness of the mutant on

Phaseolus vulgaris was checked by plant nodulation testing (Section 5.1).

5.32 Method adopted for *R. phaseoli* strain NZP 5462

YMB cultures were grown as above (Section 5.31) and plated on YMA plates containing streptomycin, rifampicin and spectinomycin. The plates were incubated at 25°C without inversion. Plates with isolated colonies were replica plated onto BHI, YMA and antibiotic plates and incubated at 25°C. The double resistant mutants obtained were verified and plant tested as above (Section 5.31).

Antibiotic resistant mutants of both strains were maintained on YMA slopes at 4°C and lyophilized and stored at 4°C in the Microbiology and Genetics Department Collection.

5.4 PRODUCTION OF INEFFECTIVE MUTANTS (Zurkowski and Lorkiewicz 1978)

Rhizobium mutants (Section 5.3) were grown in YMB (50cm^3). These cultures were used to inoculate (5cm^3) four side arm flasks containing YMB (100cm^3) which were incubated in pairs at 25°C and at 35°C.

One flask from each pair was used to follow changes in optical density during the incubation period, the other was sampled daily for viable count. Optical densities were read in a klett colourimeter fitted with a red filter (no. 66) against a YMB blank.

Colonies were selected from plates inoculated with cultures held at 35°C for 2 weeks, and tested for their ability to nodulate *Phaseolus vulgaris* (Section 5.1). Bacteria were isolated from presumptive ineffective nodules (Section 5.2) and their inabiity to nodulate effectively was confirmed (Section 5.1).

5.5 AGAROSE GEL ELECTROPHORESIS:

Horizontal gel electrophoresis was performed in agarose gels on a flat bed apparatus (McDonnell *et al*, 1977). Agarose (1.5%, Sigma, Type I) was dissolved in 200cm³ electrophoresis buffer and used to form the legs in two stages. These were allowed to set before pouring the gel itself using 100cm³ agarose at 0.5, 0.7 or 1.0% as required. The teflon comb, used to form sample wells, was inserted into the molten agarose and the gel allowed to set.

Legs for Seaplaque agarose gels were made with electrophoresis buffer which did not contain EDTA (Section 3.52).

The legs were reused many times, however the current was always passed through them in the same direction.

After setting the teflon comb was removed and samples (80-100µl) containing marker dye (0.1% SDS, 0.05% bromophenol blue in water) were added.

The legs of the apparatus were then placed in tanks containing electrophoresis buffer connected to a Shandon SAE 2761 power pack. Electrophoresis was carried out at 110V (approximately 5V per cm) for various times as required.

After electrophoresis the gel was cut out with a scalpel and stained for 20-60 mins in 100cm³ deionised water containing 1cm³ of ethidium bromide (0.04mg/cm³ solution, washed in distilled water and examined over a short wave UV transilluminator (265nm) (UV Products, California).

The gel was photographed on Kodak Tri-X 5 x 4 inch film through a Linhoff Technika lens and Kodak Wratten 23A filter. Exposure time was generally 5 mins. The film was developed for 5 mins in Kodak D-19 developer and fixed for a similar time in Ilford Hypam fixer, then washed under running tap water for 5-10 mins.

5.6 pH MEASUREMENTS AND STANDARD BUFFERS:

pH measurements were made with a Radiometer TTT1 pH meter fitted with a GK 2302C combined glass/reference electrode (Radiometer).

The electrode was prepared by filling with saturated KCl solution and soaking in 0.1M HCl for 4-6 hours at room temperature. The electrode bulb was then rinsed and soaked in fresh buffer pH 6.5 (see below) for 1 hour. The bulb was again rinsed and placed in fresh pH 6.5 buffer overnight.

This procedure was used to clean the electrode whenever the response became excessively slow or erratic.

The type C glass electrode used required correction for sodium ion errors for measurements above pH 12.0. Corrections were made according to the nomogram in Figure 3.

The following standard pH solutions were used:

pH 6.5 - Type S1001 buffer, pH 6.5 ± 0.02 at 20°C (Radiometer) was used according to the makers instructions.

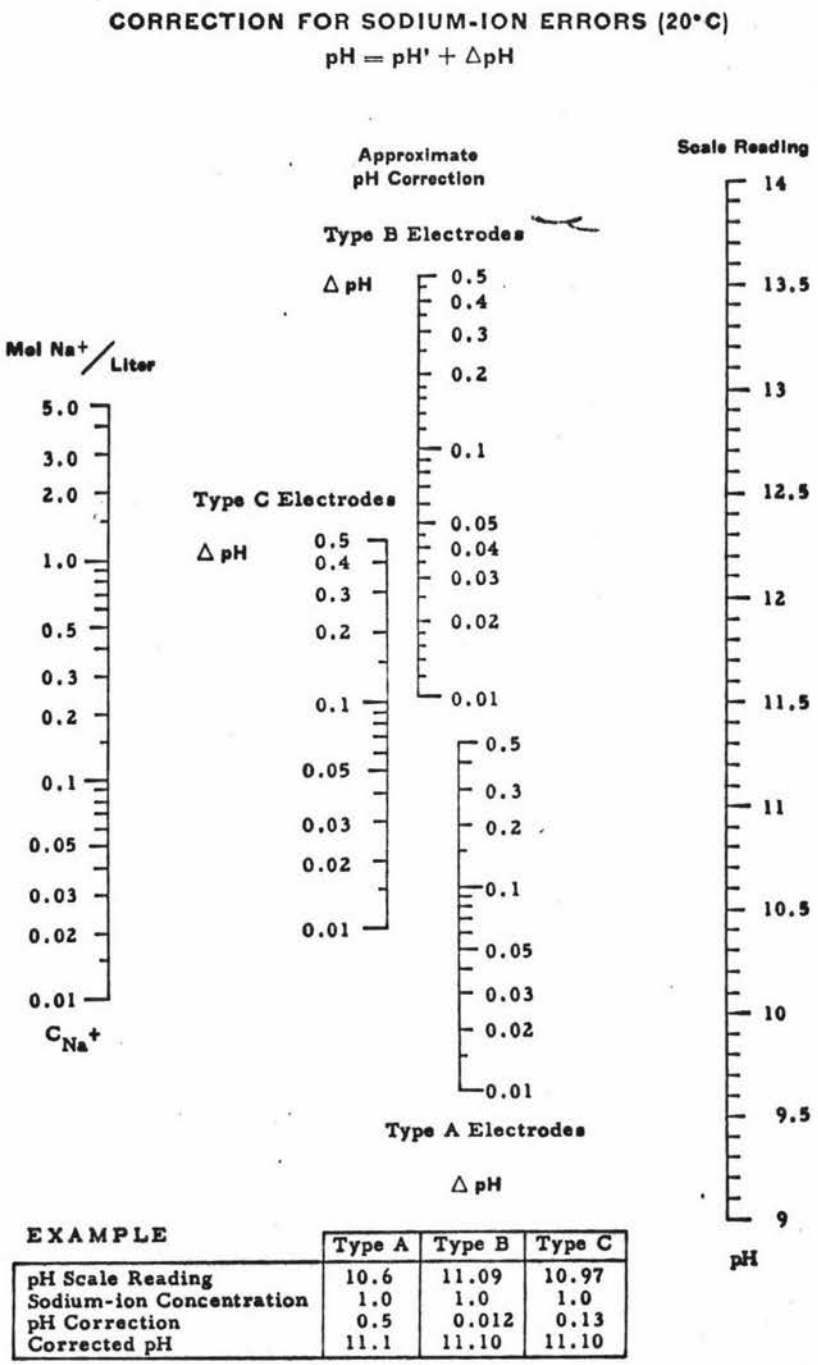
pH 9.0 - BDH no 19041 buffer, pH 9.00 ± 0.01 at 20°C was used as supplied.

pH 12.45 A saturated calcium hydroxide solution was used as a pH 12.45 standard. (Gordon and Ford, 1972).

For measurements of high pH the meter was calibrated using buffers at pH 9.0 and pH 12.45.

Further checks were made of pH in some plasmid isolations by using pH 11.0 - 13.0 (0.2 increment) indicator sticks (Merck) and pH 8-10 narrow range indicator papers (Whatman-BDH).

FIGURE 3: Nomogram for correcting sodium-ion errors



EXAMPLE	Type A	Type B	Type C
pH Scale Reading	10.6	11.09	10.97
Sodium-ion Concentration	1.0	1.0	1.0
pH Correction	0.5	0.012	0.13
Corrected pH	11.1	11.10	11.10

5.7 DETERMINATION OF DNA CONCENTRATION AND PURITY

DNA concentrations were determined by spectrophotometry at 258nm using a PYE-unicam SP1800 UV Spectrophotometer.

Sample volumes were kept small by using 0.5cm^3 quartz cuvettes to measure dilute solutions directly or by diluting the more concentrated solutions 1:200 and using a cuvette with a 1cm path length.

Absorbance was determined at 230, 258, 280 and 300nm. The Absorbances at 230 and 300nm give an indication of protein contamination, the 280 reading may be due to phenol traces or protein or both, while the DNA absorbance peak is at 258nm.

The final DNA Concentration was calculated using the formula:

$$\frac{\text{Absorbance 258nm - Absorbance 300nm}}{20} \times \text{dilution factor} = \text{DNA concentration mg/cm}^3$$

5.8 CONCENTRATION OF DNA SOLUTIONS:

5.81 Polyethylene Glycol Dialysis Method

The DNA sample to be concentrated was placed in dialysis tubing and immersed in a 50% solution of Polyethylene glycol (PEG) 20,000 molecular weight (Sigma P-2263) at 4°C until the volume had reduced to the desired level (several hours or overnight). The dialysis bag was then removed, washed in distilled water, and placed in warm distilled water for 10-20 mins to loosen the sample from the tubing. The sample was then removed with 1-2 washes of buffer.

5.82 Dialysis Against Dextran B and Carboxymethyl Cellulose

DNA samples were also dialysed against 50% Dextran (grade B) m wt 150,000-200,000 (BDH chemicals) and 10% carboxy-methyl cellulose (Sigma cat no C-4888).

5.83 Evaporation at 4°C

The DNA was placed in a dialysis membrane and hung 10-20cm in front of the fan unit in the cold room.

5.84 Ethanol Precipitation

Up to 500 µl of DNA sample was precipitated in 1.5cm³ micro centrifuge tubes by making the solution 0.4M with respect to NaCl, adding 2 volumes of 95% ethanol and holding at -70°C overnight. The sample was then centrifuged in a Beckman Microfuge for 5 mins, most of the supernatant was poured off leaving 100-200 µl in the tube. The precipitate was washed by adding 200 µl 95% ethanol (-20°C) and centrifuging for 5 mins. The supernatant was poured off leaving 50-100 µl. The precipitate was washed again with 200 µl 95% ethanol (-20°C) and all but 5-10 µl of supernatant was discarded. Residual ethanol was removed by evaporation at 37°C for 5-10 mins and the pellet resuspended in buffer or deionised water as required.

5.85 Centrifugation into a Glycerol Cushion

The DNA or lysate to be concentrated (35cm³) was centrifuged for 5 hours at 27,000 rpm 4°C in a Beckman SW27 rotor over a 1.5cm³ glycerol cushion containing 0.01M Cesium chloride and 5 µg/ml ethidium bromide. All but the last 5-6cm³ was discarded and the contents of two tubes pooled and TE buffer added to give a final volume of 35cm³.

This was centrifuged for 3 hours at 27,000 rpm 4°C over a glycerol cushion as before. The lower 5-6cm³ of concentrated DNA/glycerol was then used on an agarose gel to show the presence of plasmid bands.

5.9 PLASMID EXTRACTION METHODS

5.91 Currier and Nester (1976) Method:

1 litre of cell culture was grown on a rotary shaker in a 2l flask to a density of 50-200 Klett units (no. 66 red filter). The cells were harvested by centrifugation at 8000 rpm 10 mins in a Sorvall RC2B centrifuge fitted with a GSA rotor, and washed twice with TE buffer. After the second wash the cells were resuspended in a volume of TE buffer equal to twice the klett reading. Pre-digested pronase was added to a final concentration of 500µg/cm^3 and SDS to a final concentration of 1%. The mixture was incubated at 37°C for 60 mins to obtain a cell lysate which was sheared in 200cm^3 aliquots from 1-3 mins in a Sorvall Omni-mixer at the lowest possible speed.

The sheared lysate was placed in a 600cm^3 beaker and stirred at approx 100-150 rpm with a 2.5cm Teflon stirring bar (lowest setting on a Grant MS3 stirrer). The pH was adjusted to 12.1 to 12.3 over approx 3 mins by adding 3M NaOH dropwise (Section 5.6). After 10 mins stirring the pH of the lysate was decreased to pH 8.5-9.0 by the addition of 2M Tris buffer, pH 7.0. The solution was stirred for 3-5 mins after the final pH was reached then adjusted to 3% w/v NaCl by the addition of solid sodium chloride. An equal volume of 3% NaCl saturated phenol was added and stirred as above for 5 mins. The aqueous phase was separated by centrifuging at 6000 rpm for 5 mins in the GSA rotor then decanted into a second centrifuge bottle and residual phenol was extracted with an equal volume of 24:1 chloroform/isoamyl alcohol or 24:1 chloroform/n-butanol. The aqueous phase was separated by centrifuging at 6000 rpm for 5 mins (GSA rotor) and was decanted into another centrifuge bottle.

Magnesium chloride was added to a final concentration of 15mM and sodium phosphate buffer (pH 6.8) to a final concentration of 5mM. Ethanol (95%, 4°C, 0.7 volumes) was added and the mixture held overnight at -20°C. The Magnesium

phosphate-DNA precipitate was collected by centrifuging at 8000rpm for 20 mins. The supernatant was discarded and the pellet dissolved in 2-5cm³ of 0.1M EDTA pH 8.0 and dialysed against TES buffer for 2-24 hours. The plasmid bands were visualised by agarose gel electrophoresis.

5.92 Koekman *et al* (1980) Method:

This is essentially a modification of the Currier and Nester procedure (Section 5.91). The cells were grown, harvested and lysed according to Currierand Nester. The lysate was not sheared prior to alkali denaturation. After the final pH was reached the solution was brought to 1M NaCl final concentration by addition of solid NaCl and held at +4°C for 3-4 hours. Salt precipitated membrane-chromosomal DNA complexes were removed by centrifugation at 5000 rpm for 10 mins in a GSA rotor at 4°C. Polyethylene glycol 6000 (50%, 0.25 volumes) was added to the supernatant and this mixture was held at 4°C for 16-40 hours. DNA was precipitated by centrifugation at 8000 rpm for 20 min in a GSA rotor at 4°C, resuspended in 5-10cm³ of TE buffer, and used directly for agarose gel electrophoresis or for cesium chloride gradient centrifugation.

5.93 Schwinghamer (1980) Method:

The *Rhizobium* culture was grown into late log phase and a volume of cells equivalent to 12cm³ with an optical density of 1.0 at 600nm (Spectronic 20) were pelleted at 10,000 rpm for 15 min in a Sorvall SS 34 rotor at 0°C. The cells were resuspended in cold TS buffer and sodium dodecyl sulphate was added to a final concentration of 0.1%. This mixture was vortexed for 30 seconds then centrifuged at 10,000 rpm for 15 mins at 0°C, in a SS34 rotor. The supernatant was discarded.

The cells were resuspended in 0.4cm³ TES buffer and 0.35cm³ of a concentrated sucrose mix was added (1.6M sucrose, 0.55M

Tris 0.1M EDTA). This mixture was held at 4°C for 20 mins, after which 0.15cm³ of a 5mg/cm³ lysozyme solution in 0.05M Tris, pH8.0 was added and mixed. Then 3.6cm³ of cold 0.01M EDTA was added and the mixture incubated at 4°C for 20 mins. 2.5cm³ of 2.5% SDS was then added and the solution mixed slowly until clear. The lysate was used directly for density gradient centrifugation or run on agarose gels.

5.94 Alkaline Lysis Method; (Birnboim & Doly, 1979)

100 µl of a *Rhizobium* culture (OD 50-20 klett units, no. 66 red filter) was placed in a 400 µl Eppendof tube and centrifuged for 1 min in a Beckman Type B Microfuge. The supernatant liquid was removed with a pasteur pipette, the tube vortexed to loosen the pellet, 100 µl of alkaline SDS mixture (0.1M Tris, 3% SDS pH 12.5) added, the tube vortexed again and incubated for 30 mins at 65°C. The tube was vortexed at 15 mins and at the end of the incubation time 100 µl of a 2M Tris pH 7.0 saturated 1:1 phenol/chloroform-isoamyl alcohol (24:1) mixture was added. The tube was vortexed and centrifuged for 2 mins.

The tubes were allowed to stand for 5 min after which the supernatant liquid was removed and examined by agarose gel electrophoresis.

5.95 Casse *et al* (1979) Method:

5.951 Small Scale Method

The bacteria were grown in 50cm³ of YMB. Sodium chloride solution (5M) was added to a final concentration of 1M, and the culture was shaken vigorously for 30 mins, harvested by centrifugation at 6000 rpm for 10 mins in a Sorvall GSA rotor and washed twice with TE buffer. The lysing buffer (TE + 1% SDS) was adjusted to pH 12.45 with 3M NaOH. The pellet was resuspended in 0.5cm³ TE buffer, transferred to a 50cm³ beaker and 9.5cm³ of freshly prepared lysing buffer was added into the beaker. This mixture was stirred at 100 rpm for 90 secs., then incubated at 34°C for 20-25

mins. The pH was reduced to 8.5-8.9 by the addition of 2M Tris pH 7.0 and the mixture stirred at 100 rpm for 2 mins.

The lysate was adjusted to 3% NaCl and allowed to stand for 30 mins after which 10cm³ of 3% NaCl saturated phenol was added. The phases were mixed by stirring at 300 rpm for 10 secs then 100 rpm for 2 mins and separated by centrifugation at 5000g for 10 mins. The clear aqueous upper phase was decanted into a centrifuge tube and adjusted to 0.3M with respect to sodium acetate. Two volumes of cold (-20°C) 95% ethanol were added and the mixture held overnight at -20°C.

The precipitated DNA was recovered by centrifuging at 12000g for 20 mins at -10°C. The ethanol was decanted, the pellet dissolved in 100 µl of TES buffer pH 8.0 and examined by agarose gel electrophoresis.

5.952 Large scale method

Method 5.951 was scaled up by using 9.5cm³ lysing buffer per 0.5cm³ of cell suspension and extracting with an equal volume of phenol.

5.953 Modified large scale method

The cells were grown to an optical density of 100-150 (Klett, no 66 red filter) and washed twice in TE buffer pH 8.0, then resuspended in a small volume of TE buffer. A quantity of lysing buffer equal to twice the Klett reading was added. The mixture was then processed as for the small scale method except that the volume of phenol used was equal to that of the aqueous phase.

5.96 Anderson *et al* (1981) Method:

20cm³ of cells were harvested by centrifugation in a Sorvall SS 34 rotor at 6,500 rpm for 10 mins at 5°C in Corex tubes (30cm³). The pellet was washed with 0.9% NaCl, then with TE buffer pH 8.0 and frozen on dry ice. The pellet was thawed, 0.1cm³ of TE buffer added, and the tube vortexed gently. The cell suspension was transferred to a screw capped tube (kimax), lysozyme solution (0.1cm³, 10mg/cm³) was added and the contents mixed by gentle rotation then held on ice for 30 mins with further mixing at 10 min . intervals. A little solid pronase on the tip of a pasteur pipette was then added (or 0.1cm³ of a 10mg/cm³ solution)* mixed by rotation and the tube held on ice for 15 mins. SDS solution (10µl, 20%) was added and the tube rolled gently until the sample became very viscous, then held for 15 mins on ice. RNA ase solution (50 µl, 1mg/cm³) was added, mixed and held on ice for 15 mins.

Phenol (3cm³) saturated with TE buffer was added and the phases mixed gently for 2 mins by inverting slightly and tilting backwards and forwards and shaking gently while tilted. The phases were separated by centrifugation in a bench centrifuge (Gallenkamp), at full setting (approximately 3000 rpm) for 15 mins at 4°C (centrifuge in cold room). The clear aqueous phase was removed and examined by agarose gel electrophoresis.

5.97 Kado and Liu (1979) Method:

Cultures in YMB were incubated aerobically at 28°C for 3 days. Cell suspension (3cm³) at an OD_{600} of 0.8 was transferred to a 30cm³ corex tube and centrifuged at 6000rpm for 10 mins (Sorvall SS34 rotor). The pellet was resuspended without washing in 1.0cm³ of electrophoresis buffer (Section 3.51) and 50 µl of 0.1M EDTA added. Kado lysing solution

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* If pronase was added as a solid, 0.1cm³ distilled water was added with the phenol.

(3% SDS, 50mM Tris pH 12.6) was added (2cm³) and lysis carried out at temperatures varying from 0°C to 95°C for 30 mins. A mixture of equal parts of phenol and chloroform (6cm³) was added and the phases briefly mixed then separated by centrifugation at 8000rpm for 20 mins (Sorvall GSA rotor). The clear aqueous phase was examined by gel electrophoresis.

5.98 Eckhardt (1978) Method:

5.981 LYSOZYME MIXTURE consisted of lysozyme, 7500 U/cm³; Ribonuclease I, 0.3U/cm³; bromophenol blue (0.05%) and Ficoll 400,000 (Pharmicia) 20%, in Tris-borate buffer. The ribonuclease was dissolved in 0.4M sodium acetate buffer pH 4.0 at a concentration of 10mg/cm³, heated for 2 mins at 98°C and then added to the lysozyme mixture.

5.982 SDS MIXTURE consisted of 0.2% sodium dodecyl sulfate and 10% Ficoll 400,000 (Pharmicia) in Tris borate buffer.

5.983 Overlay mixture contained 0.2% SDS and 5% Ficoll 400,000 (Pharmicia) in Tris borate buffer.

5.984 Eckhardt method

One or two single colonies were picked with a sterile toothpick and resuspended in 15 µl of Eckhardt lysozyme mixture (Section 5.981) in the well of an agarose gel. The suspension was allowed to stand for 2-5 mins at room temperature, then 30 µl of SDS mixture (Section 5.982) was layered on top of the mixture. The two layers were very gently mixed by moving the toothpick from side to side twice. Overlay mixture (Section 5.984) (100 µl) as layered on the mixture without disturbing the viscous lysate. The gel was then electrophoresed and examined as described previously.

6. GRADIENT CENTRIFUGATION

6.1 PREPARATION OF A LINEAR SUCROSE GRADIENT

6.11 APPARATUS

Gradients were formed with a perspex block (Figure 4) containing two conical chambers A and B, connected with a tap, C. Air was bubbled through Chamber A. A pump was connected to the outlet to pump the sucrose mix into the centrifuge tube.

Formation of a 5-20% gradient in a SW41 tube: With Tap C closed 6.5cm^3 20% sucrose (Section 4.71) was pipetted into Chamber A. The tap was opened briefly to fill the interconnecting tube then closed. 5.5cm^3 of 5% sucrose solution was pipetted into Chamber B.

Air was bubbled through Chamber A to ensure mixing of the solutions. The flow of air was adjusted with clamp D. Tap C was opened, the pump started and the SW41 centrifuge tube filled, avoiding mixing in the centrifuge tube.

Other gradients were similarly formed by varying the concentration of starting solutions in chambers A and B, the most concentrated solution always being in Chamber A. (see Table V)

6.12 CENTRIFUGATION

Approximately 0.5cm^3 of DNA sample in TES buffer was layered onto the top of the gradient with a sterile pasteur pipette.

The gradients were centrifuged for various lengths of time (0.75-5.0 hrs) at 4°C and 40,000rpm in a SW41 rotor in a Beckman L5-75 ultracentrifuge. The centrifuge was allowed to slow down without the use of the brake.

FIGURE 4: Apparatus for forming sucrose gradients

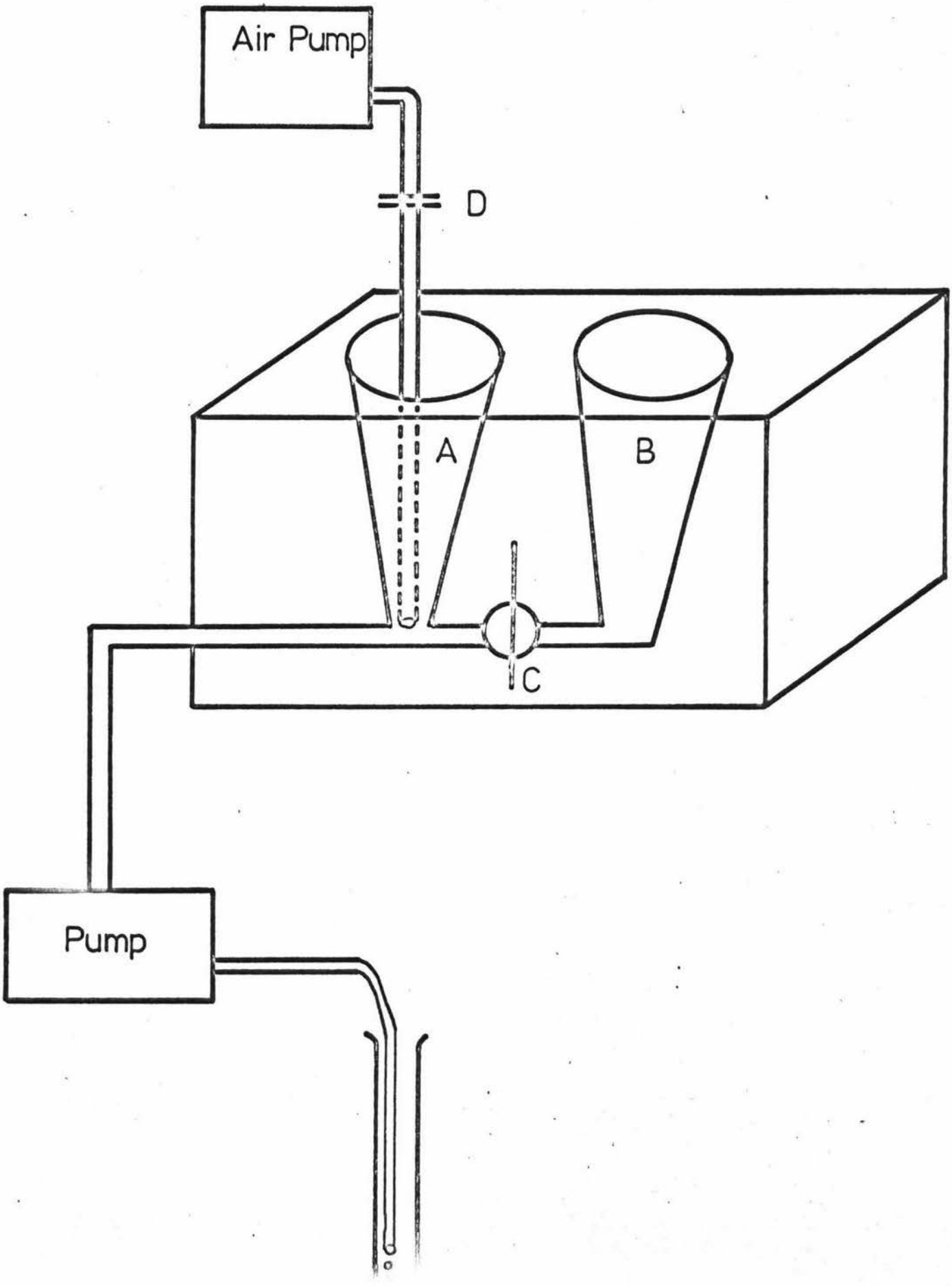


TABLE V: SUCROSE GRADIENT SOLUTIONS

% Sucrose Solution	Volume stock solution (66%) made up to 100 cm^3 total (cm^3)
5%	7.58
10%	15.15
20%	30.30
30%	45.46

6.13 FRACTIONATION

Gradients were fractionated on an Isco model 640 density gradient fractionator. Sucrose chase solution (Section 4.72) was pumped into the bottom of the tube at a flow rate of 0.5cm³ per min and 0.3cm³ fractions were collected. Fractions were monitored with an Isco absorbance/fluorescence monitor (model UA-5) at 254nm using a type 6 optical unit with the following settings; range 1.0, base 6.0, chart speed 60cm/hr. Samples of each fraction (100µl) were electrophoresed in 0.7% agarose gels to determine their plasmid and chromosomal DNA content.

6.2 CESIUM CHLORIDE-ETHIDIUM BROMIDE DENSITY GRADIENTS

Cesium chloride gradients were formed in various rotors as follows.

6.21 SW 50.1 (5.0cm³ tube capacity)

The DNA sample was brought to a total volume of 2.8cm³ with TE buffer and 3.3g cesium chloride (Sigma, optical grade) dissolved in it then 0.7cm³ of a 4mg/cm³ solution of Ethidium bromide (2, 7-Diamino-10-ethyl-9-phenyl-phenanthridinium Bromide, Sigma) was added. After addition of ethidium bromide exposure of tubes to light was avoided. The tubes were filled and balanced with paraffin oil and centrifuged at 36,000rpm for 36-60 hours at 15°-20°C in a Beckman L5-75 ultracentrifuge.

6.22 Type 50 (10cm³ tube capacity)

7.0gm of cesium chloride was dissolved in 7cm³ of DNA solution and 0.5cm³ of a 10mg/cm³ Ethidium bromide solution added as above. The tubes were centrifuged at 45,000rpm for 48-60 hours at 15°-20°C in a Beckman L2-65B, L5-75 or L8-70 ultracentrifuge.

6.23 Type 40 and 50 Ti (13.5cm³ tube capacity)
8.0gm of cesium chloride was dissolved in 8.0cm³ of DNA solution and 0.6cm³ of a 10mg/cm³ Ethidium bromide solution added as above. The tubes were centrifuged at 40,000rpm (Type 40) or 50,000rpm (50 Ti) as in Section 6.22.

6.24 Visualisation and Removal of DNA bands
Tubes were removed from rotors in a dark room illuminated by red light and DNA bands were visualised by holding approximately 5cm in front of a long wave (365nm) ultraviolet light source (UVSL-25, Ultraviolet Products Inc, San Gabriel, California). The centre plug was removed from the tube cap to permit free entry of air and the chromosomal band drawn into a 1cm³ Tuberculin syringe through a 20 gauge 1½ inch needle (Yale) inserted in the side of the tube just below the band.

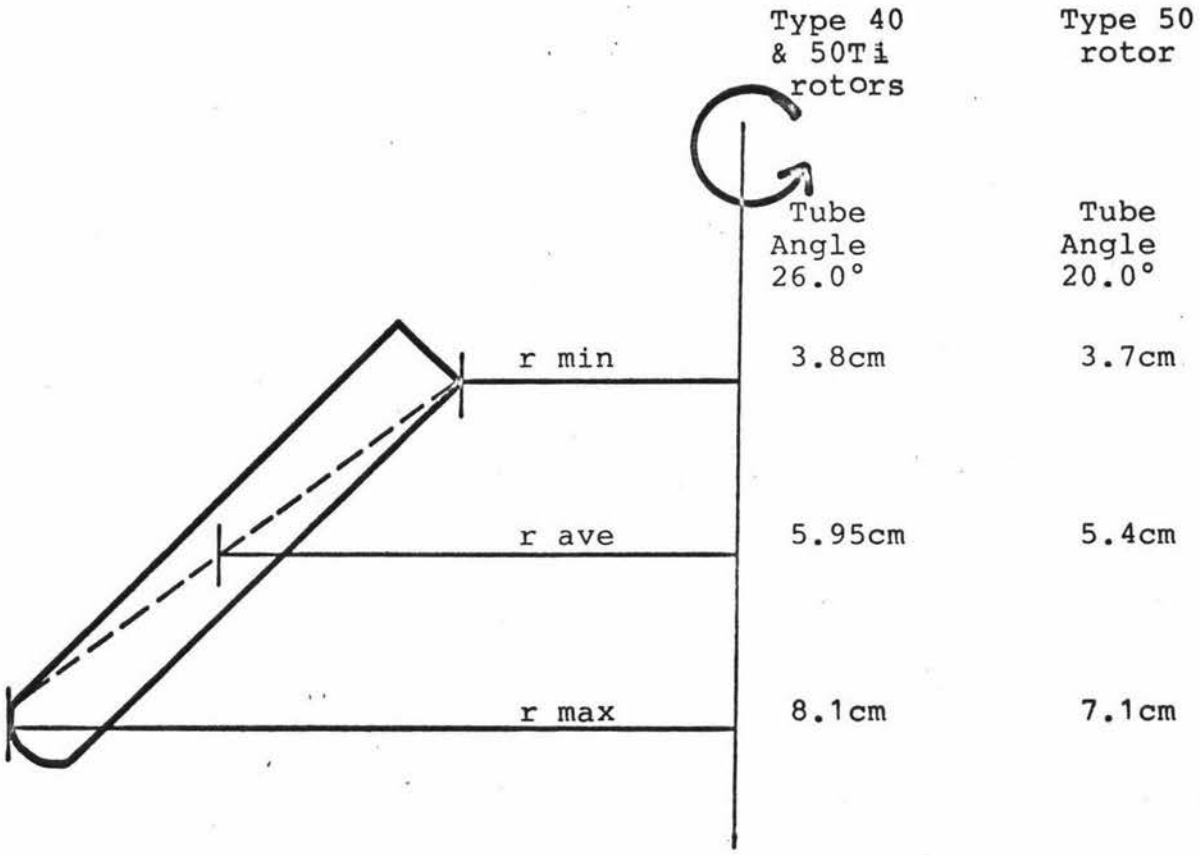
Removal of the chromosomal (upper) band first prevented contamination of the plasmid band with chromosomal DNA which was then removed as for the chromosomal band.

The needle was removed from the syringe to avoid shearing and the DNA transferred to a 1.5cm³ micro centrifuge tube. Ethidium bromide was extracted with iso-amyl or n-butyl alcohol, 2-3 extractions usually being necessary. All steps up to the removal of ethidium bromide were carried out under red light to avoid possible nicking of the plasmid DNA.

The extracted plasmid band was dialysed against Tris buffer (0.05M, pH 7.0) for 36-48 hours (3 changes, 4°C) to remove Cesium chloride. The concentration of DNA in the dialysate was determined from the absorbance at 258nm (Section 5.7).

6.25 Calculation of Theoretical Cesium Chloride Gradients (McCall & Potter, 1973)
The centrifuge tube angles and radius from the rotor centre are given in Figure 5. The density of the Cesium chloride

FIGURE 5: Centrifuge tube angles and radius in Beckman ultracentrifuge rotors



gradient, (ρ) at a point χ in the tube is given by the formula:

$$\rho = \rho_o + \frac{\omega^2}{2\beta} (\chi^2 - \chi_o^2)$$

where ρ_o is the density of the starting solution at χ_o, the isoconcentration point. β is a constant, equal to 1.19×10^2 kg^{-1} M^5 S^{-2}. The angular velocity, ω is equal to;

$$\frac{2\pi(rpm)}{60} \quad S^{-1}$$

At a rotor speed of 35,000rpm, $\omega = 3665$ S^{-1}, at 45,000rpm, $\omega = 4712$ S^{-1} and at 50,000rpm, $\omega = 5236$ S^{-1}. For example, for the type 40 and 50 Ti rotors at 35,000rpm.

$$\rho \text{ bottom} = \rho_o + \frac{\omega^2}{2\beta} (\chi^2 - \chi_o^2)$$

$$= 1.5825 + \frac{(3665)^2}{2.(1.19 \times 10^2} [(0.081)^2 - (0.0595)^2]$$

$$= 1.752g \text{ cm}^{-3}$$

$$\rho \text{ top} = \rho_o + \frac{\omega^2}{2\beta} (\chi^2 - \chi_o^2)$$

$$= 1.5825 + \frac{(3665)^2}{2.(1.19 \times 10()} [(0.038)^2 - (0.0595)^2]$$

$$= 1.463g \text{ cm}^{-3}$$

The theoretical densities at the top and bottom of cesium chloride gradients centrifuged under various conditions are given in Table VI. The information in this table shows that to obtain the widest range for the gradient it is necessary to use the Type 50 Ti rotor at maximum speed (50,000rpm).

TABLE VI: THEORETICAL DENSITY OF CESIUM CHLORIDE GRADIENTS

Rotor	rpm	density g/cm^3	
		ρ top	ρ bottom
Type 40	35,000	1.46	1.75
Type 50	45,000	1.44	1.78
	50,000	1.41	1.83
Type 50 Ti	35,000	1.46	1.75
	45,000	1.42	1.82
	50,000	1.34	1.93

7. RESTRICTION ENDONUCLEASE ASSAY METHODS

7.1 Method 1

The reaction mixture contained 25µl of the appropriate enzyme buffer (Section 3.6), 5 µl gelatin (1mg/cm^3, Davis), 10 µl enzyme diluted in deionised water as required, and 10 µl DNA solution containing 2µg DNA.

The restriction endonuclease was added last and the mixture incubated at 37°C in a waterbath for 60 mins. The reaction was stopped by the addition of 10 µl stopping reagent containing 50% sucrose, 0.1M EDTA and 0.05% bromophenol blue. The digest was electrophoresed in a 1% agarose gel at 110V until the bromophenol blue dye front had reached the end of the gel (usually about 2 hrs). The gel was stained and examined as previously described (Section 5.5).

7.2 Method 2

The reaction mixture contained 20µl 5x *Eco* RI buffer (Section 3.62), 2 µl enzyme, 2.0 µg DNA and deionised water to 100 µl. The mixture was digested at 37°C for 90 mins then at 60°C for 15-20 mins. 10 µl stopping reagent (as for method 7.1) was added and the digest electrophoresed in a 1% agarose gel at 60V for 6 hrs. The gel was stained and examined as previously described (Section 5.5).

RESULTS

1. PLANT NODULATION TESTS

Nodulation tests (Method 5.1) were carried out on the *Rhizobium* strains listed in Table VII. The appearance of the plants was examined after three weeks growth. This is shown in plate 1. The ineffective strains are already beginning to show a yellowing in appearance while the effective strains retain a healthy green colouration. After 5 weeks this difference has become even more marked and the effective and ineffective strains can be clearly distinguished in plates 2, 3 and 4. Effectiveness was assessed from the presence or absence of nodules and the top weight (Table VII).

The student's t test (Mendenhall and Ott, 1980) for comparing two population means was used to compare the mean of the negative control with each strain mean.

The formula:

$$t = \frac{\bar{y}_1 - \bar{y}_2}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

was used where $\bar{y}_1$ and $\bar{y}_2$ are the sample means, that is the mean of the negative control and the mean of the strain being compared, n_1 and n_2 are the sample sizes, that is the number of pots used to determine the mean weights and

$$S = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}$$

Where S_1 and S_2 are the sample means i.e. the standard deviations from the negative control mean and the strain mean.

TABLE VII: Effectiveness of root nodules formed on bean plants by strains of *Rhizobium* used in this investigation

Strain	No. of Pots	No. of Plants	Mean wet weight of Tops/ Pot (g)	Standard Deviation	Mean Dry weight of Tops/ Pot (g)	Standard Deviation	Appearance[1]	Nodules[2]
Negative Control	5	14	2.92	1.05	0.42	0.16	inef	-
5097	2	6	2.55	0.35	0.33	0.014	inef	-
5232	2	6	3.4	1.13	0.46	0.14	inef	-
5260	2	6	4.2	0.57	0.58	0.17	inef	-
5492	2	6	6.25	0.35	1.40	0.14	eff	+++
5459	2	6	5.4	1.56	1.22	0.50	eff	+++
F300	2	6	2.75	0.21	0.32	0.014	inef	-
F310	2	6	3.0	0.28	0.38	0.11	inef	-
5443	2	6	4.15	0.07	0.84	0.06	eff	++
5456	2	6	4.0	0.00	0.80	0.03	eff	++
5384	2	6	5.85	0.21	1.31	0.16	eff	++
5463	2	6	4.0	0.00	0.74	0.03	eff	+++
5462	2	6	5.95	0.21	1.15	0.07	eff	++

[1] APPEARANCE. The tops of the plants were assessed 'inef' if they were yellow in colouration or 'eff' if they appeared a healthy green colour.

[2] NODULES. The root systems were assessed for the presence (+) or absence (-) of nodules.

PLATE 1: Plant nodulation tests. Appearance of plants after three weeks growth. Strains are (left to right); Negative Control, *R. phaseoli* NZP 5459, NZP 5492, NZP 5260, NZP 5097, NZP 5232.

PLATE 2: Plant nodulation tests. Appearance of plants after five weeks growth. Strains are (left to right); *Rhizobium* sp. NZP 5384, *R. phaseoli* NZP 5260, NZP 5492, *Rhizobium* sp. NZP 5463, *R. phaseoli* NZP 5232, negative control.

1

2

PLATE 3: Plant nodulation tests. Appearance of plants after five weeks growth. Strains are (left to right); *Rhizobium* sp. NZP 5462, *R. phaseoli* F300, *Rhizobium* sp. NZP 5443, *R. phaseoli* F310, *Rhizobium* sp NZP 5456.

PLATE 4: Plant nodulation tests. Appearance of plants after five weeks growth. Strains are (left to right); *R. phaseoli* NZP 5097, NZP 5459, *Rhizobium* sp NZP 5384, *R. phaseoli* NZP 5260, NZP 5492.

3

4

The student t test was applied with $(n_1 + n_2 - 2)$ degrees of freedom and a rejection region of 0.05.

The t values obtained from this analysis are given in Table VIII.

A 95% confidence interval requires that t values below 2.015 be rejected as there being no statistically significant difference between the test strain and the negative control.

From the wet top weight data strains 5492, 5459, 5384 and 5462 show a significant increase in shoot weight and were thus effective in fixing nitrogen in the bean plant.

The dry top weight results divide the strains into three groups, ineffective strains, that is those with no significant difference from the negative control, a second group which shows a significant difference with 95% confidence and a third group which shows a significant difference with greater than 99% confidence.

The information for appearance, nodulation and shoot dry weight indicates that the strains used in this study had the following effectiveness on bean plants; Ineffective *R. phaseoli* strains NZP 5097, NZP 5232, NZP 5260, F300 and F310; Partially effective, *Rhizobium* sp NZP 5443, NZP 5456, NZP 5463; Effective *R. phaseoli* strains NZP 5492, NZP 5459, and *Rhizobium* sp NZP 5462 and NZP 5384. The effectiveness of the strains is diagramatically illustrated in Figure 6.

2. Selection of spontaneous antibiotic resistant mutants of *Rhizobium phaseoli* which retain the ability to nodulate beans.

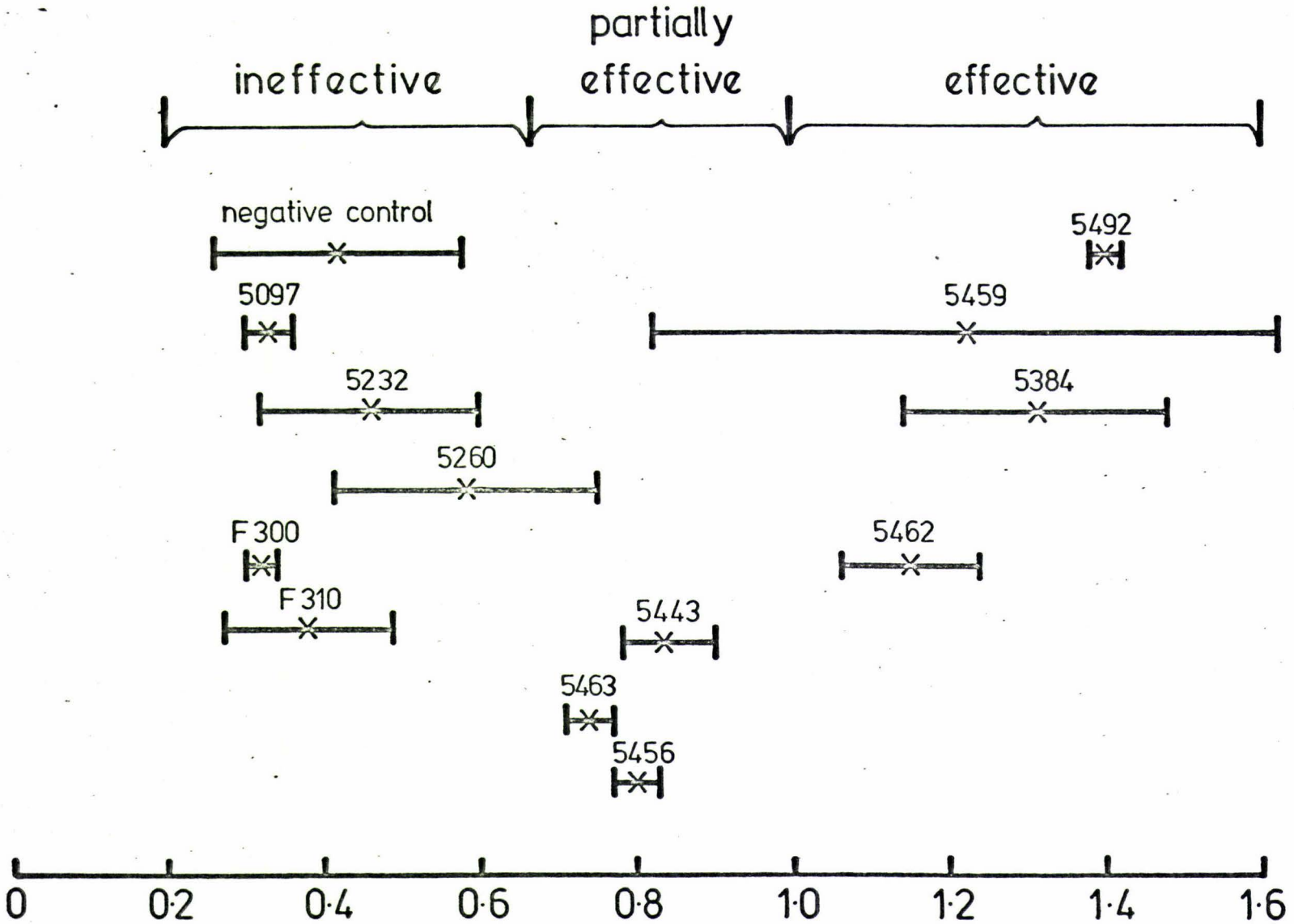
TABLE VIII: Statistical comparison by student's t test[2] of the top dry weights from plants inoculated with *Rhizobium* strains used in this investigation and the top dry weight of uninoculated control plants[1]

Strain (NZP)	Sample Size n_2	WET WEIGHTS OF TOPS Sample Mean $\bar{y}_2$	Standard Deviation s_2	t	DRY WEIGHTS OF TOPS Sample Mean $\bar{y}_2$	Standard Deviation s_2	t
5097	2	2.55	0.35	0.465	0.33	0.014	0.75
5232	2	3.4	1.13	0.53	0.46	0.14	0.31
5260	2	4.2	0.57	1.56	0.58	0.17	1.46
5492	2	6.25	0.35	4.19	1.40	0.014	8.2
5459	2	5.4	1.56	2.53	1.22	0.50	3.64
F300	2	2.75	0.21	0.22	0.32	0.014	0.83
F310	2	3.0	0.28	0.10	0.38	0.11	0.31
5443	2	4.15	0.07	1.56	0.84	0.06	2.8
5456	2	4.0	0.00	1.37	0.80	0.03	2.71
5384	2	5.85	0.21	3.71	1.31	0.16	5.56
5463	2	4.0	0.00	1.37	0.74	0.03	2.29
5462	2	5.95	0.21	3.84	1.15	0.07	4.87

[1] Values for the uninoculated control, $n_1 = 5$; for the wet top weights, $\bar{y}_1 = 2.92$ $s_1 = 1.05$; for the dry top weights, $\bar{y}_1 = 0.42$, $s_1 = 0.16$

[2] For the t test; degrees of freedom = 5,
$t_{0.05} = 2.015$ t values greater than 2.015 indicate significant difference from the negative control

FIGURE 6: The effectiveness of *Rhizobium* strains used in this investigation


ineffective
partially effective
effective
negative control
5492
5097
5459
5232
5384
5260
F 300
5462
F 310
5443
5463
5456
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
Mean dry weight of tops per pot (gm)
(error bars show standard deviation)

To ensure that ineffective mutants (method 5.4) were derivatives of the parent strain and not contaminants it was necessary to mark the strains used. This was done by selecting for spontaneously occurring double antibiotic resistant mutants then obtaining ineffective mutants with the same antibiotic resistance from these effective resistant strains.

2.1 Nalidixic acid

Nalidixic acid at a concentration of 100 µg/cm³ was not effective against the *Rhizobium* strains examined. It therefore was not useful in selecting the spontaneous mutants required.

2.2 *Rhizobium phaseoli* NZP 5492 (Method 5.31)

A culture of strain NZP 5492 (0.5cm³) plated on YMA supplemented with streptomycin (Method 2.52) gave 5 to 10 colonies per plate. Sixty (60) streptomycin resistant colonies were verified by transferring to streptomycin supplemented YMA however none of these were able to grow on rifampicin supplemented plates.

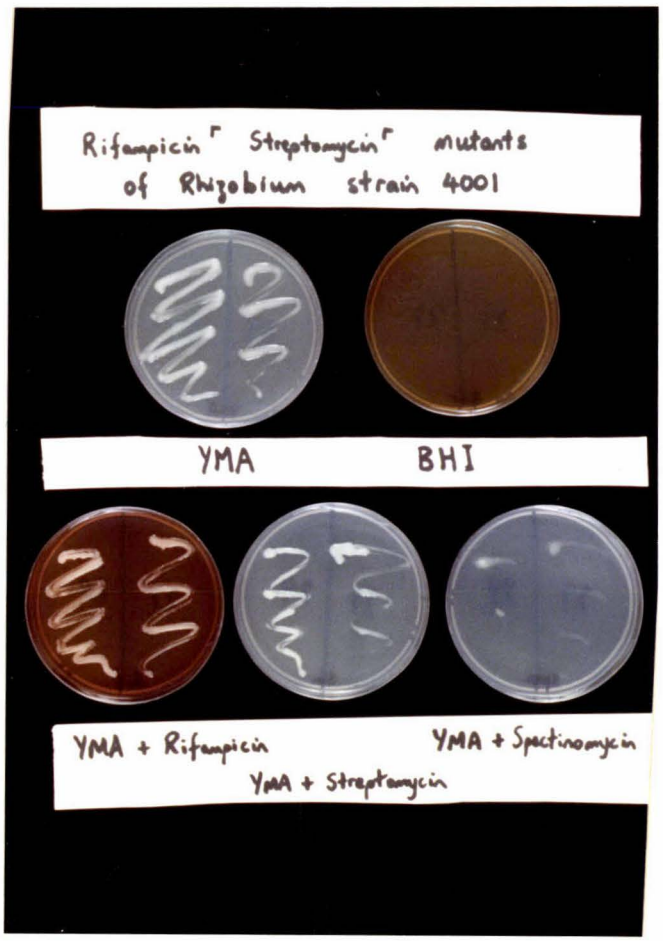
Growth of the streptomycin resistant mutants in broth culture followed by plating 0.5cm³ samples onto antibiotic plates gave 19 streptomycin resistant rifampicin resistant (sm^r rif^r) double mutants and 4 streptomycin resistant spectinomycin resistant (sm^r $spec^r$) double mutants. These strains did not grow on BH1 and appeared to be typical rhizobia in morphology and gram strain reaction. Examples of the mutants obtained are shown in plates 5 and 6.

2.3 *Rhizobium* sp. NZP 5462 (Method 5.32)

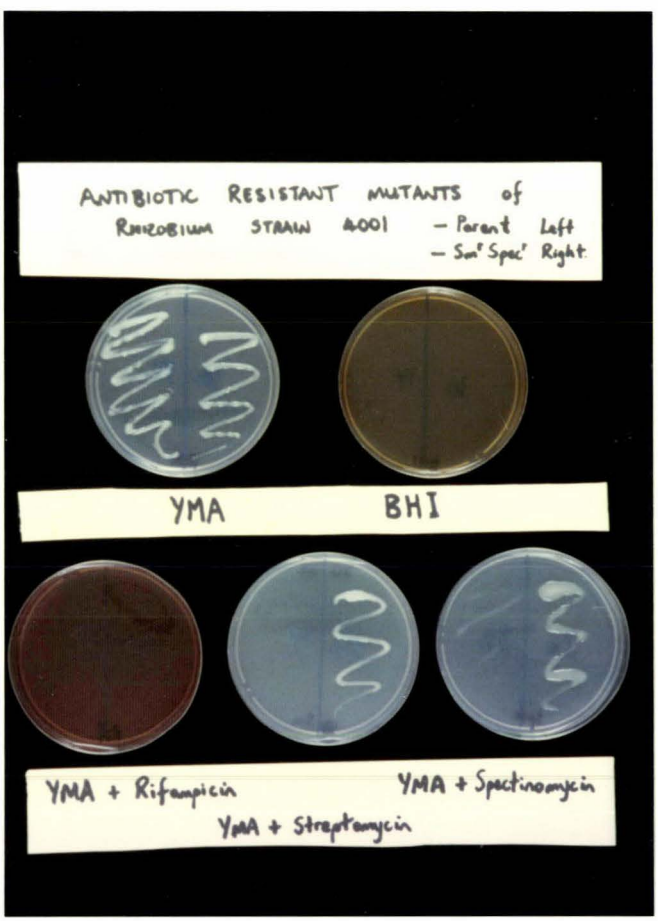
Rhizobium sp NZP 5462 was plated on YMA supplemented with streptomycin (Method 2.52), rifampicin (Method 2.51) and

PLATE 5: Streptomycin resistant rifampican resistant mutants of *Rhizobium phaseoli* strain NZP 5492 (4001)

PLATE 6: Streptomycin resistant spectinomycin resistant mutant of *Rhizobium phaseoli* strain NZP 5492 (4001)


Rifampicin^r Streptomycin^r mutants
of Rhizobium strain 4001
YMA
BHI
YMA + Rifampicin
YMA + Spectinomycin
YMA + Streptomycin

5


ANTIBIOTIC RESISTANT MUTANTS of
RHIZOBIUM STRAIN 4001 – Parent Left
– Sm^r Spec^r Right
YMA
BHI
YMA + Rifampicin
YMA + Spectinomycin
YMA + Streptomycin

6

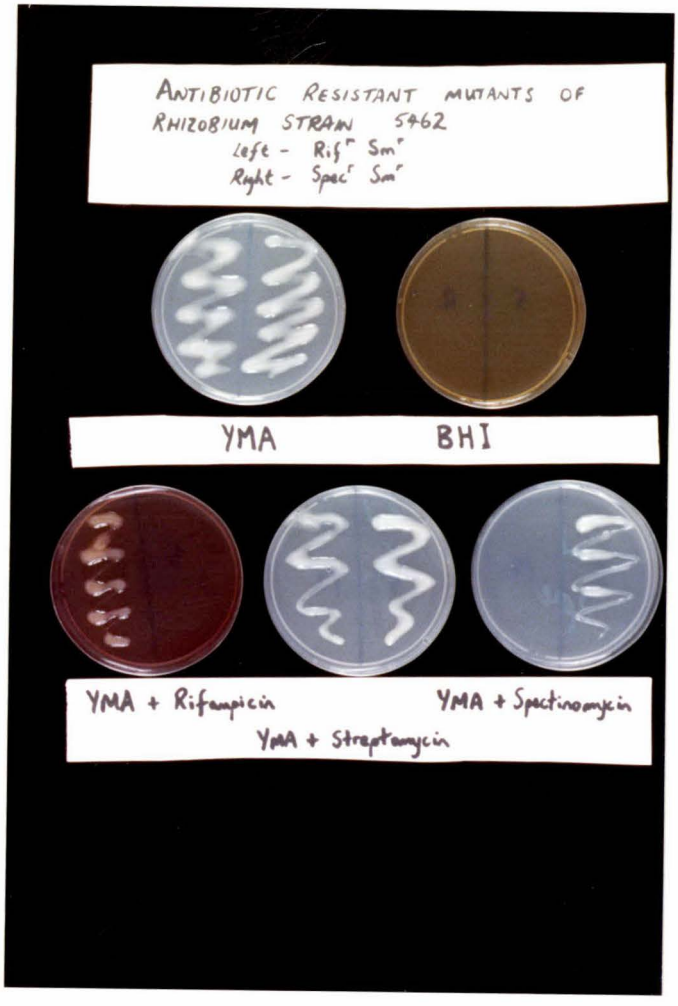
spectinomycin (Method 2.53). Ten plates of each type were inoculated and 7 spectinomycin resistant ($Spec^r$) colonies, 94 rifampicin resistant (Rif^r) colonies and 166 streptomycin resistant (Sm^r) colonies were obtained. The resistant colonies were replica plated onto YMA supplemented with the antibiotics as above. Of the 166 streptomycin resistant colonies, 2 were also resistant to spectinomycin and 11 to rifampicin. Of the seven colonies resistant to spectinomycin 5 were also resistant to rifampicin and 5 to streptomycin. Of the 94 rifampicin resistant colonies 66 were resistant to streptomycin and none to spectinomycin. All *Rhizobium* sp NZP 5462 mutants were typical in morphology and gram stain reaction. Examples of the mutants obtained are shown in Plate 7.

2.4 The ability of the mutant strains to nodulate and fix nitrogen on bean plants

It was necessary to verify that (a) the parent strains; *Rhizobium phaseoli* NZP 5492 $Sm^S Rif^S Spec^S$ and *Rhizobium* sp NZP 5462 $Sm^S Rif^S Spec^S$ were effective, that is significantly greater top weights than the negative control and (b) that the antibiotic resistant mutants selected were as effective as the parent strains, that is that the top weights were not significantly less than the parent strains.

The results of the nodulation tests are given in Table IX. The statistical comparison of the results was made using the student t test (Table X). A 95% significant difference between the nitrogen deficient control and the test strain is given by t greater than 2.447 and a 99% significant difference by $t > 3.143$. The results in Table X show that the parent strains and mutants are all effective with greater than 99% confidence.

PLATE 7: Antibiotic resistant mutants of *Rhizobium* sp. strain NZP 5462.


ANTIBIOTIC RESISTANT MUTANTS OF
RHIZOBIUM STRAIN 5462
Left - Rif^r Sm^r
Right - Spec^r Sm^r
YMA
BHI
YMA + Rifampicin
YMA + Spectinomycin
YMA + Streptomycin

7

TABLE IX: Effectiveness of root nodules formed on bean plants by *Rhizobium phaseoli* NZP 5492 and *Rhizobium* sp NZP 5462 and antibiotic resistant mutants obtained from these strains

Strain	No. of Pots	No. of Plants	Mean wet weight of Tops/ Pot (g)	Standard Deviation	Mean Dry weight of Tops/ Pot (g)	Standard Deviation	Appearance[1]	Nodules[2]
Negative Control	5	15	3.04	0.40	0.48	0.09	inef	-
5492 Parent	3	9	6.33	0.95	1.23	0.32	Eff	++
5492 Sm^rRif^r	38	111	6.26	1.34	1.30	0.30	Eff	++
5492 Sm^rSpec^r	7	19	6.14	1.25	1.26	0.27	Eff	++
5462 Parent	2	6	6.8	0.14	1.35	0.07	Eff	++
5462 Sm^rSpec^r	5	14	5.4	1.32	0.90	0.30	Eff	++
5462 Sm^rRif^r	15	44	5.98	1.01	1.17	0.34	Eff	++

[1] APPEARANCE
[2] NODULES Refer to Table VII footnotes

TABLE X: Statistical comparison by Student t test[2] of the top weights of beans inoculated with *R. phaseoli* NZP 5492, *Rhizobium* sp. 5462, their antibiotic mutants and the top weight of uninoculated beans in nitrogen deficient growth conditions[1]

Strain (NZP)	Sample Size n_2	WET WEIGHTS OF TOPS Sample Mean $\bar{y}_2$	Standard Deviation s_2	t	DRY WEIGHTS OF TOPS Sample Mean $\bar{y}_2$	Standard Deviation s_2	t
NZP 5492 antibiotic sensitive parent	3	6.33	0.95	7.0	1.23	0.32	5.14
NZP 5492 Sm^rRif^r	38	6.26	1.34	5.27	1.30	0.30	6.3
NZP 5492 Sm^rSpec^r	7	6.14	1.25	5.25	1.26	0.27	6.0
NZP 5462 antibiotic sensitive parent	2	6.8	0.14	12.5	1.35	0.07	11.6
NZP 5462 Sm^rSpec^r	5	5.4	1.32	3.8	0.90	0.30	3.0
NZP 5462 Sm^rRif^r	15	5.98	1.01	6.3	1.17	0.34	4.3

[1] Values for the nitrogen deficient control, $n_1 = 5$; for the wet top weights, $\bar{y}_1 = 3.04$, $s_1 = 0.40$; for the dry top weights $\bar{y}_1 = 0.48$, $s_1 = 0.09$

[2] See footnotes to Table VIII

3. Isolation of ineffective mutants from antibiotic resistance marked clones

Method 5.4 was used in an attempt to isolate a nodulation deficient (nod-) or ineffective (fix-) mutant from *R. phaseoli* NZP 5492 Rif^r Sm^r and *Rhizobium* sp. NZP 5462 Sm^r $Spec^r$.

There was no detectable difference in the viable count or the optical density (Klett red filter No. 66) (Figs 7 and 8) between the cells grown at 25°C and those at 35°C. The gradual increase over the second week of sampling is not due to viable cells which remained constant over this period. It is probably due to the formation of slime and other factors affecting the measurement of optical density.
Cell numbers were initially 3×10^8 for NZP 5492 and 6×10^9 for NZP 5462. The cultures peaked at 10^{12} cells after 7 days for NZP 5492 and 10^{13} cells after 6 days for NZP 5462. There was no difference in the number of days taken to reach maximum viable count or in the maximum viable count between the two temperature treatments. The viable counts remained relatively constant from days 8 to 14 with no difference between the cultures at each incubation temperature.

After approximately 10 days a number of smaller colonies began to appear on the dilution plates of the cultures incubated at 35°C. The frequency of small colonies was approximately 10-15% after 14 days. These colonies were gram negative rods with the appropriate antibiotic resistance.

Fifteen small colonies resistant to rifampicin and streptomycin from strain NZP 5492 and 8 small colonies resistant to streptomycin and spectinomycin from strain NZP 5462 were screened on *Phaseolus vulgaris*. The effectiveness

FIGURE 7: Changes in optical density in cultures of *Rhizobium phaseoli* NZP 5492 incubated at 25°C and 35°C

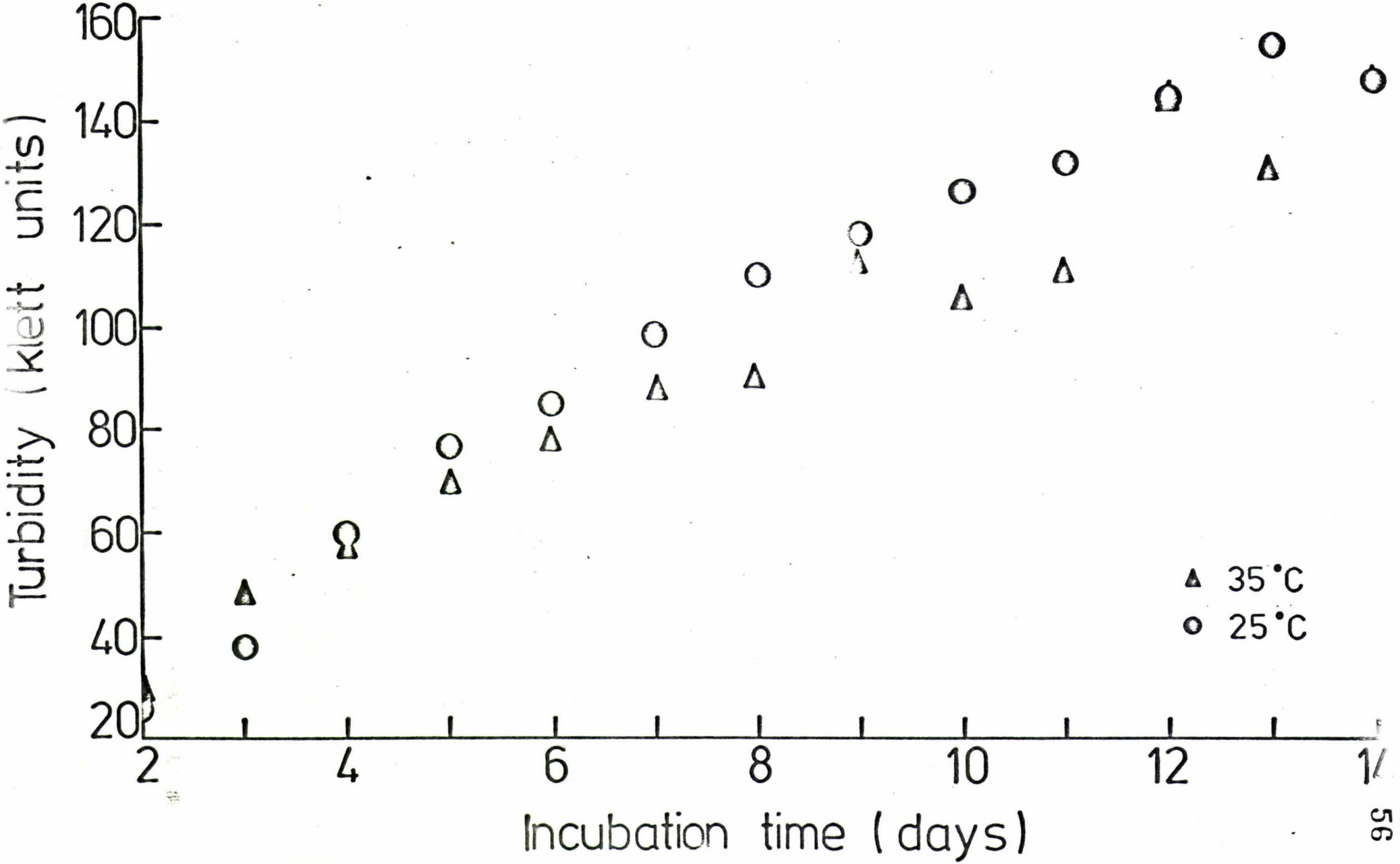
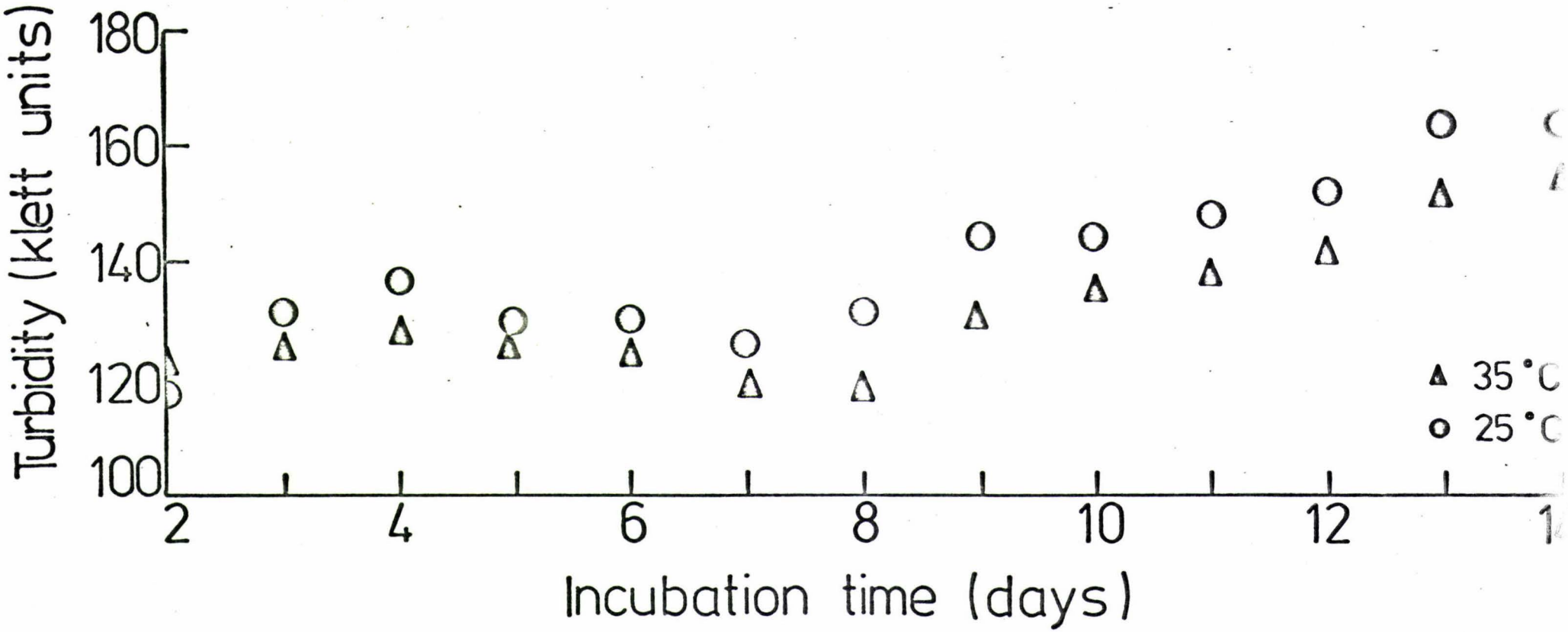

Turbidity (klett units)
160
140
120
100
80
60
40
20
2
4
6
8
10
12
Incubation time (days)
35 °C
25 °C

FIGURE 8: Changes in optical density in cultures of *Rhizobium* sp. NZP 5462 incubated at 25°C and 35°C.


Turbidity (klett units)
180
160
140
120
100
2
4
6
8
10
12
Incubation time (days)
35 °C
25 °C

of small colony variants was compared with that of the antibiotic resistant mutant and wild type parent (Table XI). The NZP 5462 Sm^r $Spec^r$ small colony mutants were at least as effective as the wild type strain and no ineffective (fix-) or non-nodulating (nod-) mutants were obtained. The NZP 5492 Sm^r Rif^r mutants were only partially effective compared to the parent strains and negative controls. Statistical analysis of the results is given in Table XII. All of the small colony mutants produced pink effective nodules. However, one pot inoculated with a small colony mutant from NZP 5492 Sm^r rif^r had some small white nodules (Plate 8) which were disected and found to be white throughout, lacking any red pigment.

Rhizobia were isolated from these presumptive ineffective nodules (Method 5.2) and gram negative Sm^r rif^r bacilli were recovered. Six similar nodules were examined and 38 Sm^r rif^r colonies which were obtained from the nodules were grown in broth culture and tested for effectiveness on beans. Seven of the 38 strains tested appeared to be ineffective. In plate 9 a presumptive ineffective pot B5/1 is compared to the negative and positive controls and the parent strain NZP 5492 Sm^r Rif^r. In plate 10 the presumptive ineffective mutant pot B5/1 is compared with an apparantly effective pot B5/8 obtained from a small colony variant in the same way. Table XIII gives the effectiveness data for the two pots. The statistical analysis (Table XIV) indicates that NZP 5492 Sm^r Rif^r B5/1 is ineffective whereas strain NZP 5492 Sm^r Rif^r B5/8 is effective.

Pot B5/8 contained plants which had red/brown effective nodules while pot B5/1 contained plants which had large green nodules (Plate 11) and some small white nodules (Plate 12).

The green nodules when dissected were green throughout (Plate 13) compared to the effective nodules which were

TABLE XI: Effectiveness of root nodules formed on bean plants by *Rhizobium phaseoli* NZP 5492, NZP 5492 Sm^r Rif^r, small colony variants of NZP 5492 Sm^r Rif^r and *Rhizobium* sp. NZP 5462, NZP 5462 Sm^r $Spec^r$ and small colony variants of NZP 5462 Sm^r $Spec^r$.

Strain	No. of Pots	No. of Plants	Mean wet weight of Tops/ Pot (g)	Standard Deviation	Mean Dry weight of Tops/ Pot (g)	Standard Deviation	Appearance[1]	Nodules[2]
Negative Control	2	5	4.8	0.42	0.75	0.06	inef	-
NZP 5492 Parent	3	9	6.33	0.95	1.23	0.32	Eff	+++
NZP 5492 $Sm^r rif^r$	38	111	6.26	1.34	1.30	0.30	Eff	+++
NZP 5492 $Sm^r rif^r$ small colonies	17	47	5.51	1.25	0.97	0.25	Eff	+
NZP 5462 Parent	2	6	6.8	0.14	1.35	0.07	Eff	++
NZP 5462 $Sm^r Spec^r$	5	14	5.4	1.32	0.90	0.30	Eff	++
NZP 5462 $Sm^r Spec^r$ small colonies	8	21	5.6	1.03	0.98	0.23	Eff	++

[1] APPEARANCE
[2] NODULES

See footnotes to Table VII

TABLE XII: Statistical comparison by student t test[2] of the top weights of beans inoculated *R. phaseoli* NZP 5492, NZP 5492 Sm^rRif^r, NZP 5492 Sm^rRif^r small colony variants, *Rhizobium* sp NZP 5462, NZP 5462 Sm^rSpec^r, NZP 5462 Sm^rSpec^r small colony variants and the top weights of uninoculated beans in nitrogen deficient growth conditions[1]

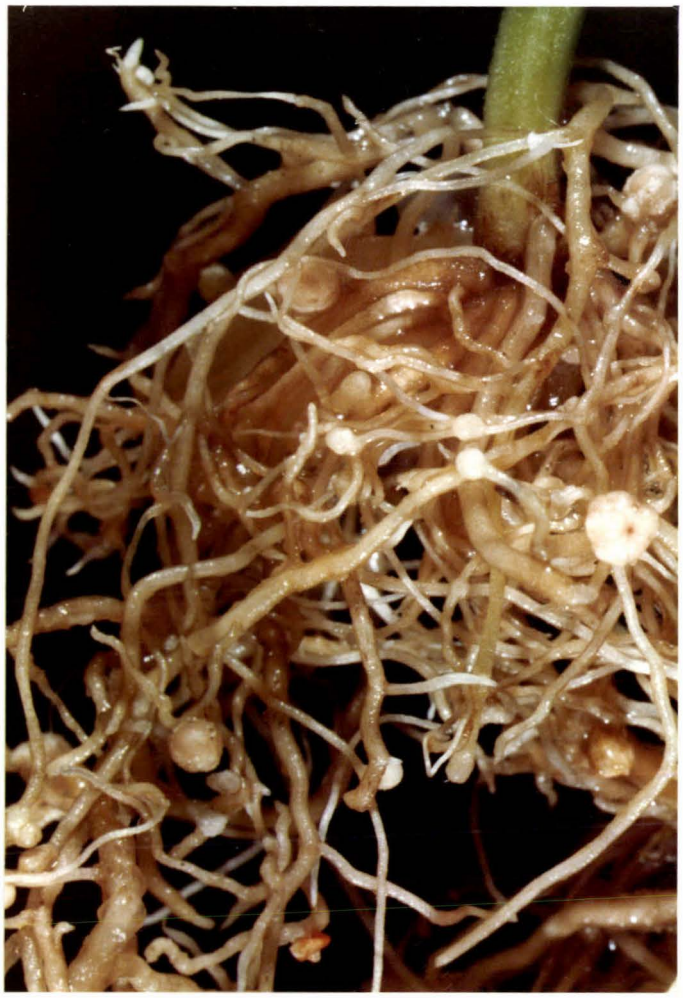
Strain (NZP)	Sample Size n_2	WET WEIGHTS OF TOPS Sample Mean $\bar{y}_2$	Standard Deviation s_2	t	DRY WEIGHTS OF TOPS Sample Mean $\bar{y}_2$	Standard Deviation s_2	t
NZP 5492 Parent	3	6.33	0.95	7.0	1.23	0.32	5.14
NZP 5492 Sm^rRif^r	38	6.26	1.34	5.27	1.30	0.30	6.3
NZP 5492 Sm^rRif^r small colony variant	17	5.51	1.25	4.1	0.97	0.25	4.1
NZP5462 Parent	2	6.8	0.14	12.5	1.35	0.07	11.6
NZP 5462 Sm^rSpec^r	5	5.4	1.32	3.8	0.90	0.30	3.0
NZP 5492 Sm^rSpec^r small colony variant	8	5.6	1.03	5.22	0.98	0.23	4.55

[1] Values for the nitrogen deficient control, n = 5; for the wet top weights, $\bar{y}_1 = 3.04$, $s_1 = 0.40$; for the dry top weights, $\bar{y}_1 = 0.48$, $s_1 = 0.09$

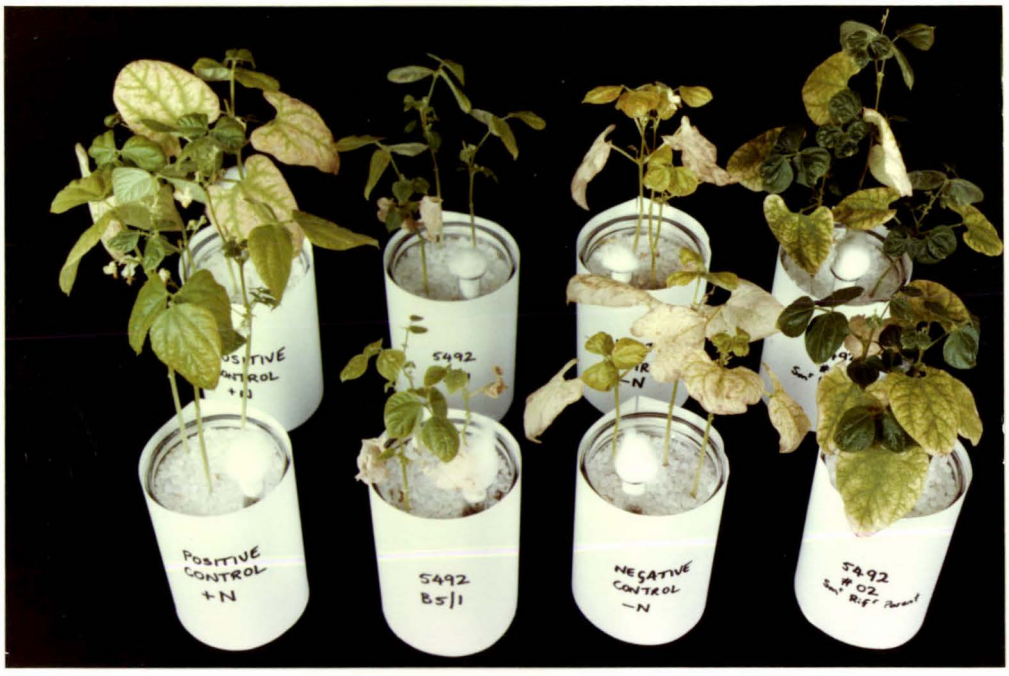
[2] see footnotes to Table VIII

PLATE 8: Root system from a bean plant incubated with *Rhizobium phaseoli* strain NZP 5492 Sm^r Rif^r, showing small white nodules.

PLATE 9: Comparison of strain NZP 5492 B5/1 Sm^r Rif^r (presumptive ineffective mutant) with the parent strain NZP 5492 Sm^r Rif^r and the positive and negative controls.



8



9

TABLE XIII: Effectiveness of root nodules formed on bean plants by *Rhizobium phaseoli* NZP 5492 Sm^rRif^r B5/1 and NZP 5492 Sm^rRif^r B5/8

Strain	No. of Pots	No. of Plants	Mean wet weight of Tops/ Pot (g)	Standard Deviation	Mean Dry weight of Tops/ Pot (g)	Standard Deviation	Appearance[1]	Nodules[2]
Negative Control (-N)	4	12	4.25	1.06	0.74	0.14	inef	-
Positive Control (+N)	4	12	9.82	0.52	1.88	0.18	eff	-
NZP 5492 Sm^rRif^r B5/1	2	6	4.69	0.98	0.68	0.05	inef	+
NZP 5492 Sm^rRif^r B5/8	2	6	7.1	0.14	1.36	0.15	eff	++

[1] APPEARANCE

[2] NODULES

See footnote to Table VII

TABLE XIV: Statistical comparison by student t test[2] of the top weights of beans inoculated with *R. phaseoli* NZP 5492 Sm^rRif^r B5/1, NZP 5492 Sm^rRif^r B5/8 and the top weights of uninoculated beans in nitrogen deficient growth conditions[1]

Strain (NZP)	Sample Size n_2	WET WEIGHTS OF TOPS Sample Mean $\bar{y}_2$	Standard Deviation s_2	t	DRY WEIGHTS OF TOPS Sample Mean $\bar{y}_2$	Standard Deviation s_2	t
NZP 5492 Sm^rRif^r B5/1	2	4.69	0.98	0.4	0.68	0.05	0.48
NZP 5492 Sm^rRif^r B5/8	2	7.1	0.14	3.56	1.36	0.15	4.77

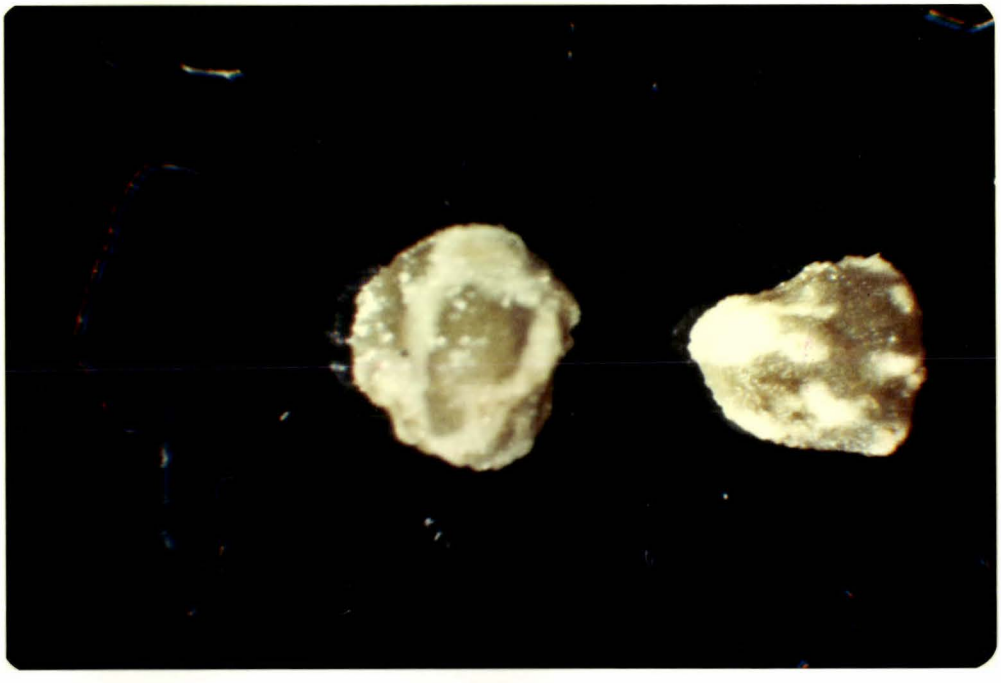
[1] Values for the nitrogen deficient control, $n_1 = 4$; for the wet top weights, $\bar{y}_1 = 4.25$, $s_1 = 1.06$; for the dry top weights, $\bar{y}_1 = 0.74$, $s_1 = 0.14$

[2] See footnotes to Table VIII

PLATE 10: Comparison of ineffective pot B5/1 and effective pot B5/8 and the positive and negative controls.

PLATE 11: Large green nodules from bean roots in pot B5/1.

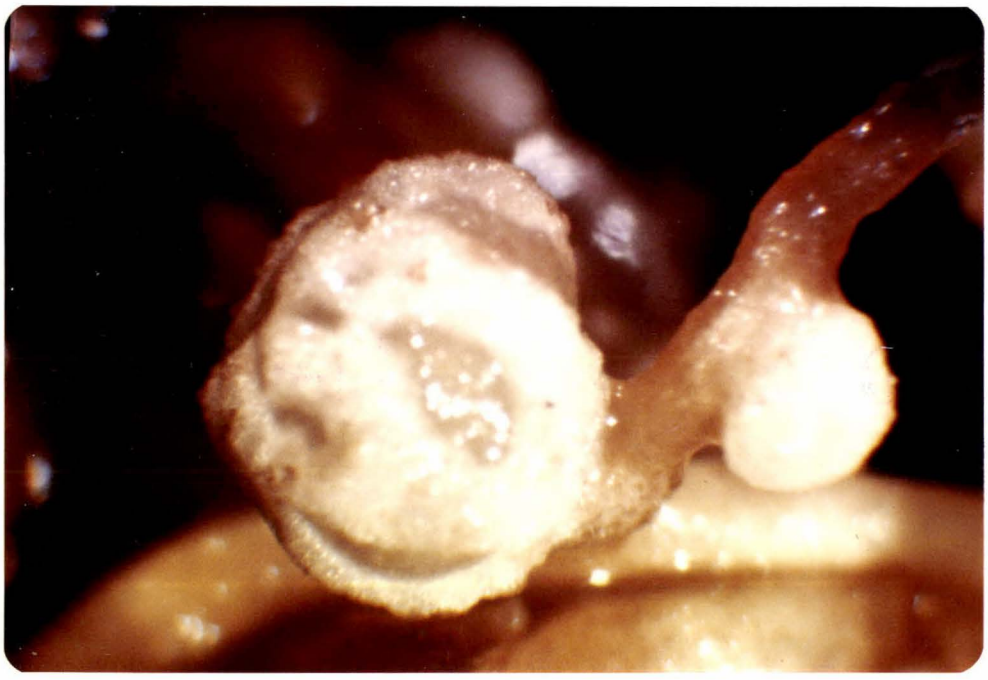
10



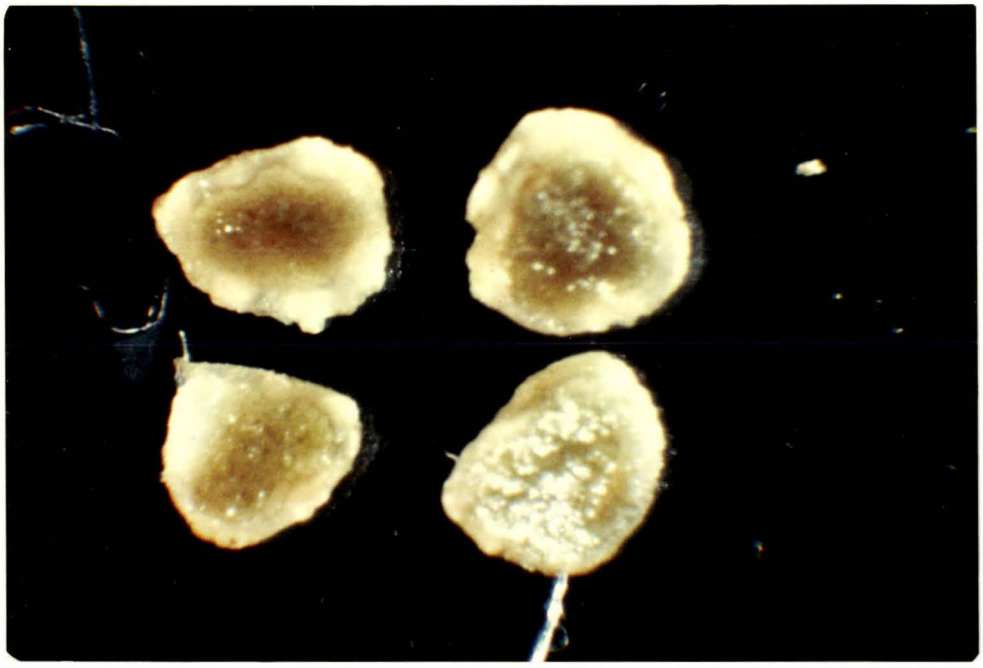
11

PLATE 12: Large green and small white nodules on the roots of bean plants in pot B5/1.

PLATE 13: Cross section of green nodules from pot B5/1.



12



13

red/brown inside (Plate 14). The three nodule types, white, green and brown are compared together in Plate 15. The red pigmentation is presumed to be leghaemoglobin, and is absent in the green and white nodules.

Smears of the fluid from green and red/brown nodules were stained with crystal violet for 60 secs. The red/brown effective nodules (Plate 16) appeared to have considerably more bacteroids than the green ineffective nodules (Plate 17).

A clone isolated from a green nodule produced in pot B5/1 was designated NZP 5492 B5/1 and a clone isolated from a red/brown nodule produced in pot B5/8 was designated NZP 5492 B5/8. The plasmid patterns of these strains were compared in later experiments.

4. *Rhizobium phaseoli* strains NZP 5479 and NZP 5547

Strains NZP 5479 and NZP 5547, a non-nodulating single colony isolate obtained from strain NZP 5479 were obtained from Dr D.B. Scott.

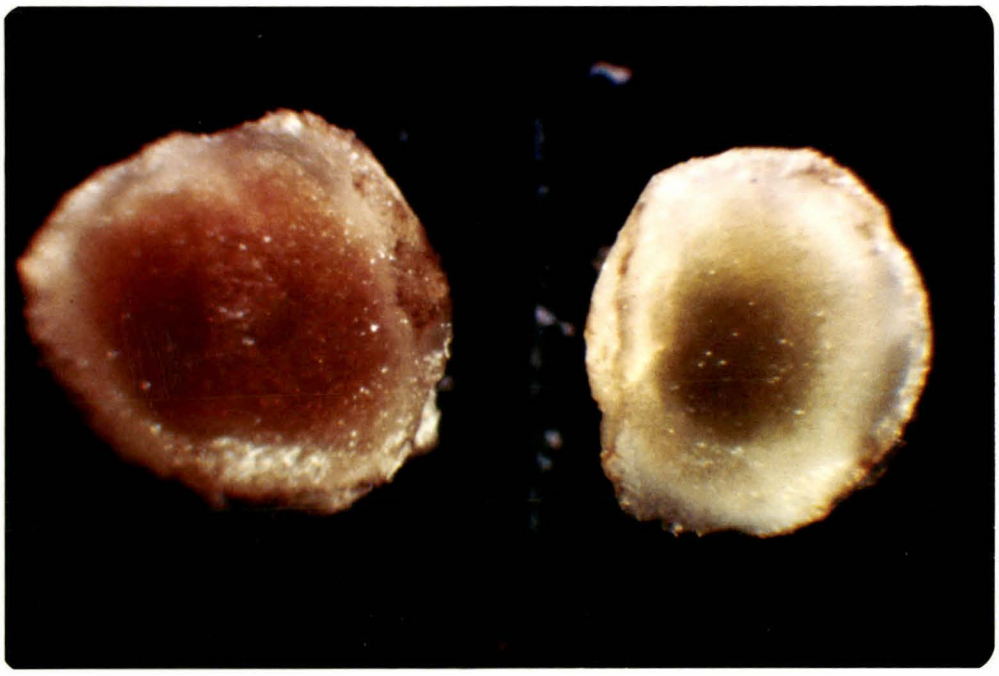
The effectiveness of these strains on beans was tested (Method 5.1). The plants are shown in Plate 18 where strain NZP 5479 is a healthy green colour and strain NZP 5547 is pale yellow similar to the negative control.

Data for the effectiveness of these strains on beans is given in Table XV. Statistical analysis of this data (Table XVI) shows that strain NZP 5479 is effective with greater than 99% confidence whereas strain NZP 5547 is completely ineffective.

Examination of the root systems shows that strain NZP 5479 produces typical red/brown effective nodules (Plate 19) while strain NZP 5547 is completely non-nodulating (Plate 20).

PLATE 14: Cross section of a red brown effective nodule from pot B5/8 showing red pigmentation compared to the green ineffective nodules from pot B5/1.

PLATE 15: Cross section comparing red/brown effective nodules, green and white ineffective nodules.



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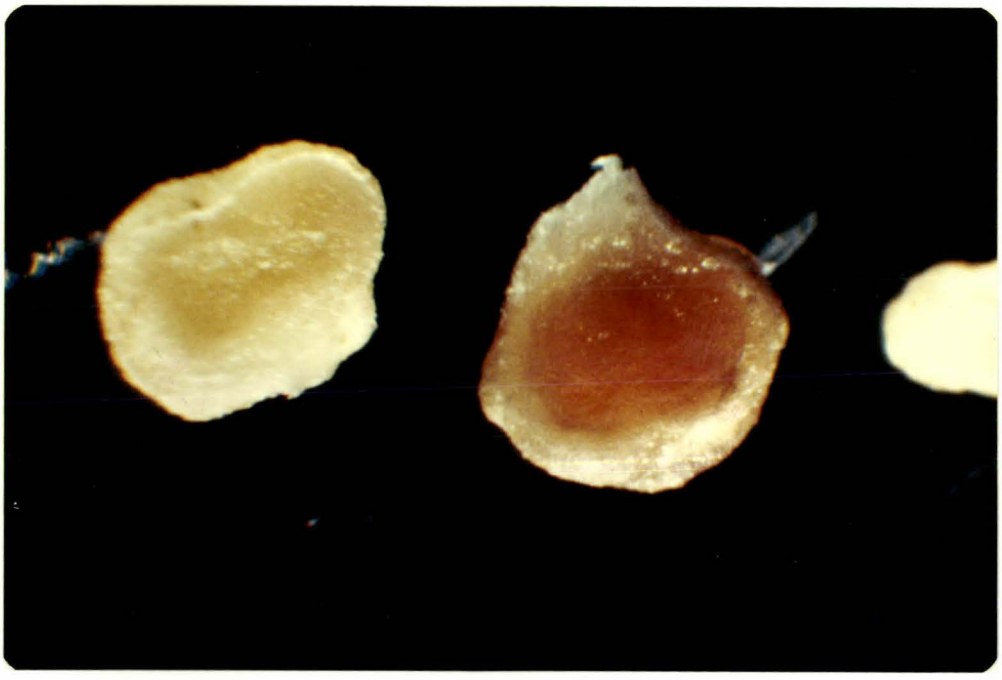

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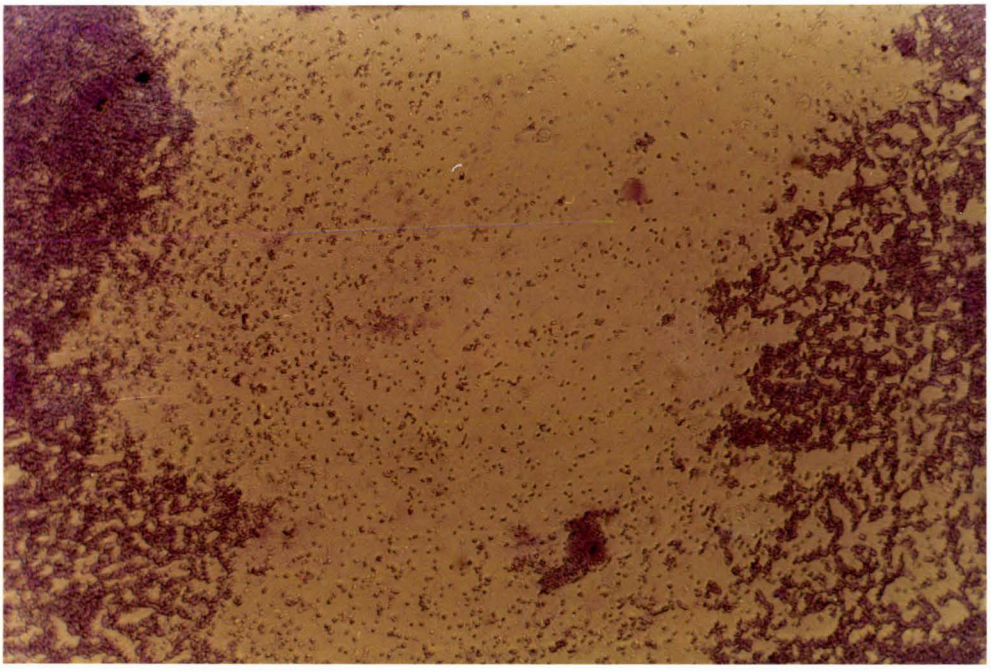
PLATE 16: Bacteria isolated from red/brown effective nodules (pot B5/8). Magnification 300x.

PLATE 17: Bacteria isolated from green ineffective nodules (pot B5/1). Magnification 300x.



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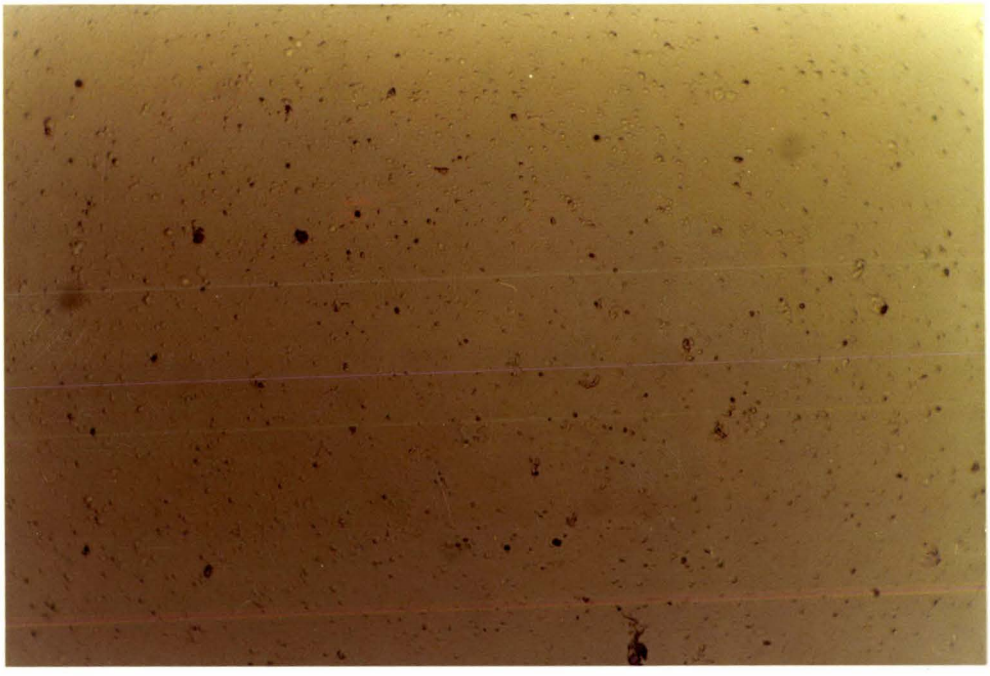

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PLATE 18: Bean plants inoculated with *Rhizobium phaseoli* strains NZP 5479 and NZP 5547 and the positive (+N) and negative (-N) control plants.

18

TABLE XV: Effectiveness of root nodules formed on bean plants by *Rhizobium phaseoli* strains NZP 547[illegible] and NZP 5547.

Strain	No. of Pots	No. of Plants	Mean wet weight of Tops/ Pot (g)	Standard Deviation	Mean Dry weight of Tops/ Pot (g)	Standard Deviation	Appearance[1]	Nodules[2]
Negative Control (-N)	4	12	4.25	1.06	0.74	0.14	inef	-
Positive Control (+N)	4	12	9.82	0.52	1.88	0.18	eff	-
NZP 5479	2	5	6.6	0.85	1.29	0.3	eff	++
NZP 5547	2	6	4.9	1.3	0.84	0.24	inef	-

1 APPEARANCE

2 NODULES See footnote to Table VII

TABLE XVI: Statistical comparison, by student t test[2] of the top weights of beans inoculated with *R. phaseoli* strains NZP 5479 and NZP 5547 and the top weights of uninoculated beans in nitrogen deficient growth conditions[1]

Strain (NZP)	Sample Size n_2	WET WEIGHTS OF TOPS Sample Mean $\bar{y}_2$	Standard Deviation s_2	t	DRY WEIGHTS OF TOPS Sample Mean $\bar{y}_2$	Standard Deviation s_2	t
NZP 5479	2	6.6	0.85	3.70	1.29	0.3	3.1
NZP 5547	2	4.9	1.3	0.61	0.84	0.24	0.62

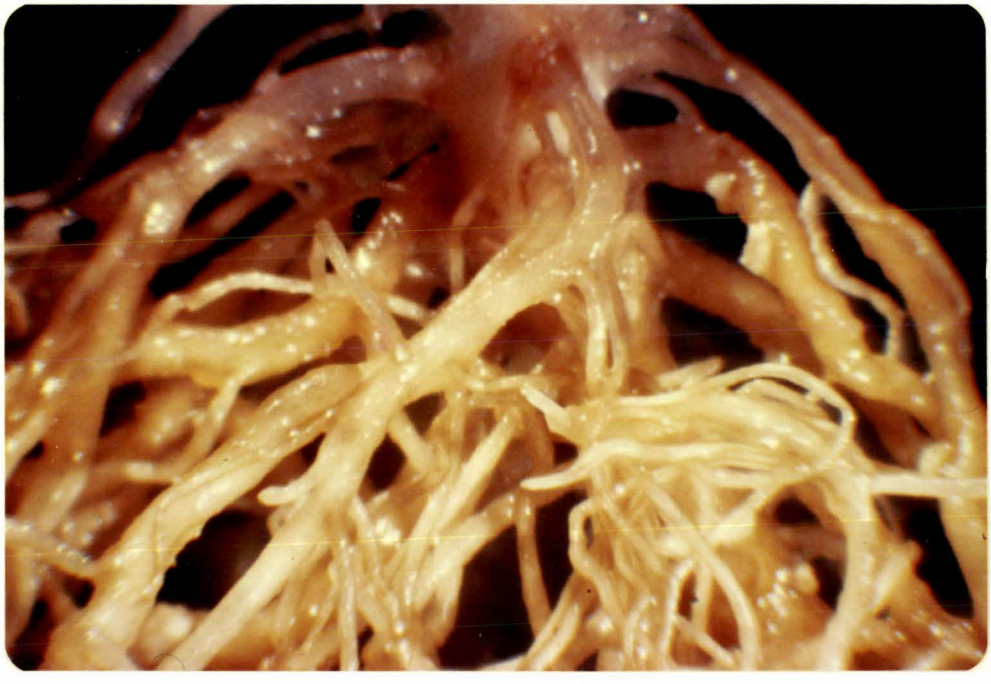
[1] Values for the nitrogen deficient control, $n_1 = 4$; for the wet top weights, $\bar{y}_1 = 4.25$, $s_1 = 1.06$; for the dry top weights, $\bar{y}_1 = 0.74$, $s_1 = 0.14$

[2] See footnotes to Table VIII

PLATE 19: Root system from a bean plant inoculated with *R. phaseoli* NZP 5479.

PLATE 20: Root system from a bean plant inoculated with *R. phaseoli* NZP 5547.

19



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The plasmids from these strains were compared in later experiments.

5. PLASMID EXTRACTION METHODS

Comparisons were made between the plasmid methods described in the methods section with the object of finding a method which would; (a) detect as many plasmids as possible in each strain and

(b) provide sufficient plasmid DNA to permit further characterisation by restriction enzyme analysis.

Since many of the gels did not have molecular weight markers the plasmid bands on different gels were compared by calculating the ratio of the distance moved by the plasmid band to the distance moved by the linear chromosomal band. This distance is referred to as the "relative mobility" of the plasmid band. Comparison of the relative mobility of a plasmid enabled comparisons to be made between gels which had been run for different lengths of time without molecular weight markers.

5.1 Alkaline lysis method

This method used 0.1cm^3 portions of the strain being examined. Lysis was achieved by treatment with SDS at high pH (12.5). The released plasmid was then extracted with phenol/chloroform isoamyl alcohol. We performed the method (Method 5.94) on a number of *R. phaseoli* strains. Plasmid bands were observed for strains NZP 5492, NZP 5097 and NZP 5260. Plate 21 shows the results obtained. The number of bands observed and their relative mobility is given in Table XVII. A band of the same relative mobility was observed in each strain.

TABLE XVII:

Strain	Number of plasmid bands obtained	Relative mobility of plasmid band compared to linear DNA
NZP 5492	1	0.48
NZP 5097	2	0.45, 0.49
NZP 5260	3	0.48, 0.54, 0.58

5.2 Anderson's (1981) Method

We used a modification of this method by D.B. Scott (Method 5.96) which has been used successfully on *Rhizobium* strains. Plasmid bands were obtained from a number of *Rhizobium phaseoli* strains. These are illustrated in Plate 22. *E. coli* R-100-1 (60 megadaltons) is included for molecular weight comparison.

Two plasmid bands were reproducibly obtained by this method for strains NZP 5097 and NZP 5492. These two strains were used to compare lysis on ice with lysis at room temperature. The results obtained (Plate 23) demonstrate that when lysis is carried out at room temperature the gel becomes overloaded with linear chromosomal DNA, masking the plasmid bands.

Homology group 2 strains NZP 5462 and NZP 5456 were compared using this method (Plate 24). Each strain had 1 plasmid band, the relative mobilities being NZP 5462 0.49 and NZP 5456 0.47.

These relative mobilities are the same as those obtained for group 1 strains with the previous method. Restriction enzyme digests are needed to compare these plasmids of similar molecular size.

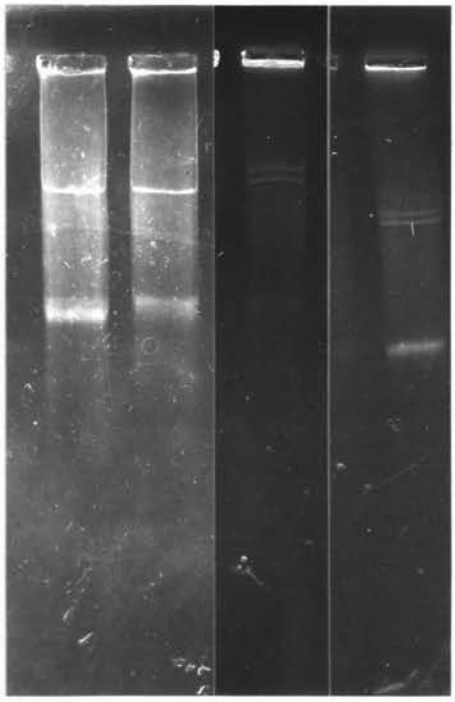
PLATE 21: Plasmid DNA obtained by the alkaline lysis method.

Tracks 1 and 2 NZP 5492
Track 3 NZP 5097
Track 4 NZP 5260

Electrophoresis was at 110V for 4 hours in a 0.7% gel.

PLATE 22: Plasmids obtained by Anderson's method.

Track 1 NZP 5260, 3 bands;
Track 2 NZP 5492, 2 bands;
Track 3 NZP 5232, 3 bands;
Track 4 NZP 5459, 1 band;
Track 5 NZP 5097, 2 bands;
Track 6 F310, 2 bands;
Track 7 *E. coli* R100-1



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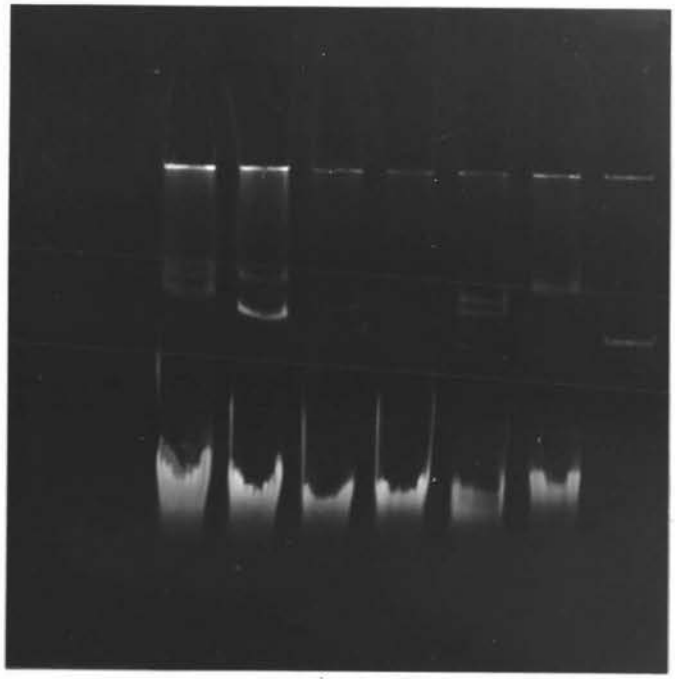

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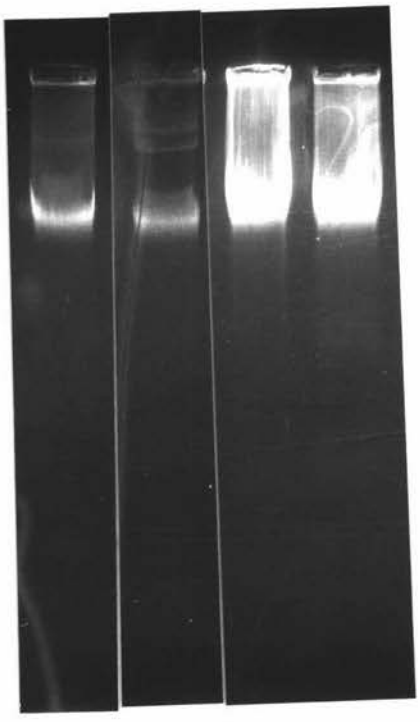
PLATE 23: Comparison of lysis temperature; Anderson method.

Track 1 NZP 5097 lysis on ice
Track 2 NZP 5492 lysis on ice
Track 3 NZP 5097 lysis at room temperature
Track 4 NZP 5492 lysis at room temperature

PLATE 24: Group 2 strains extracted by Anderson's method.

Track 1 strain NZP 5462
Track 2 strain NZP 5456



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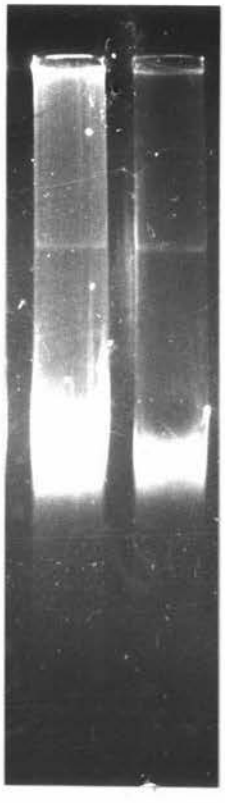

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5.3 Currier and Nester (1976) Method:

The method (Method 5.91) consisted of seven main steps. These were cell growth and harvest, cell lysis, shearing of the viscous lysate, alkali denaturation at pH 12.4, neutralization to pH 8.5 - 9.0, removal of the denatured chromosomal DNA by phenol-chloroform extraction at 3% NaCl concentration and precipitation and recovery of the plasmid.

Alteration of the lysis conditions from 37°C for 60 mins to 50°C for 15 mins did not reduce the plasmid yield. The lysate could be stored overnight at 4°C without noticeable plasmid loss. If the lysate turned brown, plasmids could not be isolated. The length of the shearing step varied according to the viscosity of the lysate. Shearing times ranged from 90 seconds to 3 minutes for very viscous lysates, which were sheared for the shorter time and the viscosity reexamined before shearing for the second 90 seconds. Excessive shearing resulted in the selective loss of large molecular weight plasmids.

The high viscosity of even the sheared lysate interferred with precise adjustment of the pH during the alkali denaturation step. The eventual yield of plasmid is dependent on the length of the shearing step, the amount and vigour of stiring and the pH reached in the alkali denaturation and neutralization steps.

Precipitation of the recovered plasmid DNA with magnesium chloride and sodium phosphate was replaced with precipitation by 0.3M sodium acetate (Garfinkel and Nester 1980).

This method gave successful isolation of plasmid bands from many *Rhizobium phaseoli* strains from both homology group 1 and group 2. Plate 25 shows a typical extraction from strain NZP 5492, giving rise to two bands. The molecular weights were estimated from the *E. coli* markers. Plate 26 also shows plasmids isolated by this method.

PLATE 25: Plasmids from NZP 5492 extracted by the method of Currier & Nester.

Track 1: *E. coli* R6K 26mD

Track 2: *E. coli* R100-1 60mD

Track 3: Vacant

Tracks 4 & 5: NZP 5492; estimated molecular weights of bands a) 89mD

b) 158mD

0.5% agarose gel electrophoresed 5 hours at 110V.

PLATE 26: Plasmids isolated by the method of Currier and Nester.

Tracks contain (left to right) *Agrobacterium tumefaciens* C58 (130mD) *Rhizobium phaseoli* U45 (101mD), *Rhizobium phaseoli* NZP 5097, *Rhizobium phaseoli* NZP 5492.

The 0.5% agarose gel was electrophoresed for 3 hours at 110V.

PLATE 27: Plasmids from NZP 5492 extracted by

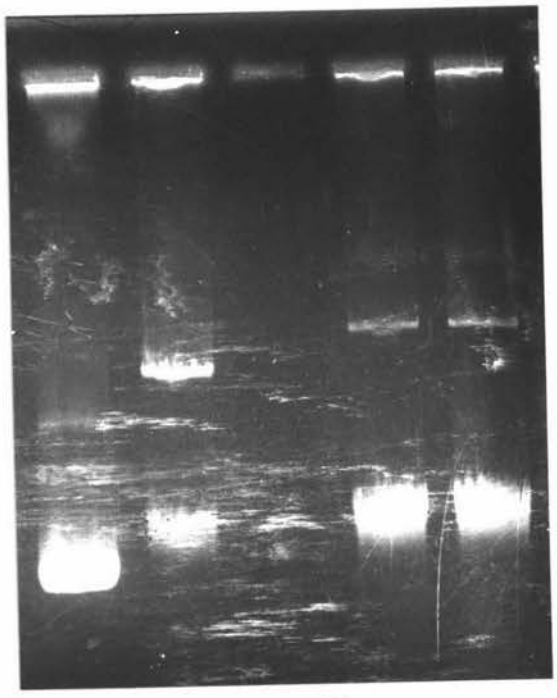
(a) Currier & Nester method

(b) Koekman method

Track 2 = (a)

Track 3 = (b)

The 1.0% agarose gel was electrophoresed for 4 hours at 110V.



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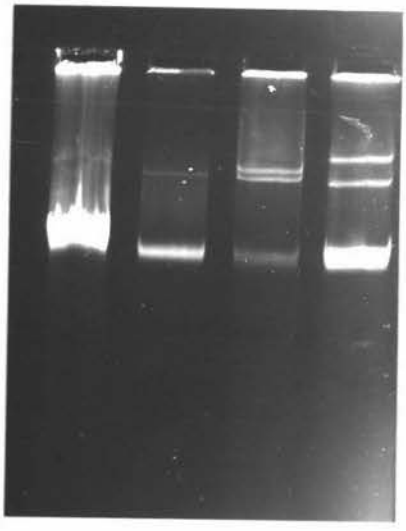

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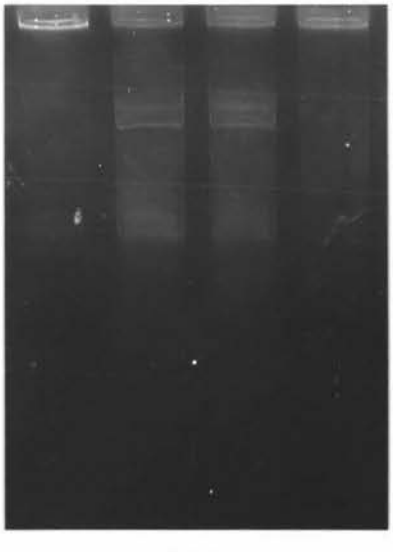


27

5.4 Koekman (1980) Method:

One of the problems with the method of Carrier and Nester (Section 5.91) is the removal of the denatured chromosomal DNA by a phenol-chloroform treatment. This requires extracting about 200 cm^3 of alkali denatured and neutralized lysate with equal volumes of firstly phenol and then chloroform. Because of the relatively large volumes involved this step is relatively awkward and expensive to perform. The Koekman variation of Currier and Nesters method (Method 5.92) harvests, lyses, shears and alkali denatures the culture in exactly the same way as the Currier and Nester method. After alkali denaturation the Currier and Nester method adjusts the lysate to 3% NaCl and then phenol-chloroform extracts. The Koekman variation is to adjust the lysate to 1M NaCl which precipates the membrane/chromosomal complex. This is then centrifuged from the solution. The DNA macromolecules are then precipitated by the addition of Polyethylene glycol 6000 instead of the ethanol precipitation of the Currier and Nester method. The resulting macromolecule precipitate is treated in the same way with both methods.

The Koekman variation was preferable in terms of time taken to perform the method and the avoidance of the use of phenol and chloroform.

The method gave rise to the same number of plasmid bands as the Currier and Nester method. The bands migrated to exactly the same position on an electrophoresis gel. Plate 27 illustrates the identical results obtained from the two methods with strain NZP 5492.

The control of pH during the alkali denaturation and neutralization steps were identical to that for the Currier and Nester method however the response of the pH electrode to changing pH is much slower in the more viscous unsheared Koekman lysate than it is in the sheared lysate used in the Currier and Nester procedure.

5.5 Kado and Liu (1979) Method:

The method of Kado and Liu (1979) (Method 5.97) was both quick and simple to perform and produced sufficient plasmid DNA to permit further investigation. It was necessary to find the optimum conditions, that is those giving maximum plasmid yield with the minimal amount of chromosomal DNA in the preparation, for the particular strains which were used in this investigation.

5.51 EFFECT OF CELL NUMBERS The concentration (optical density at 600nm) and the volume of cell suspension used determine the total number of cells lysed. In practice it was found easier to grow the cells to a standard optical density, for example 0.8 absorbance units at 600nm and then to vary the volume spun down to obtain a pellet. The effect obtained by such variance of cell numbers is illustrated in Plate 28 and Figure 9; which show preparations from the lysis of various volumes of strain NZP 5459 at 62°C for 30 mins.

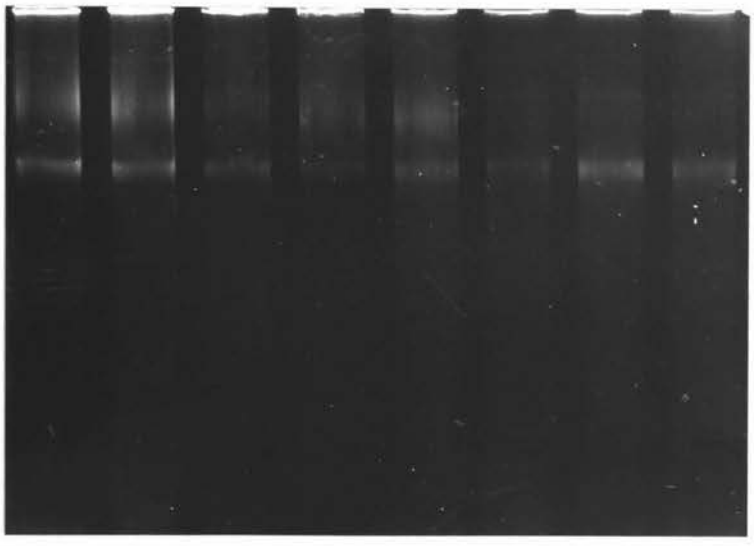
Low cell numbers do not provide enough DNA to visualise the plasmid band. Note the 'smeared region' between the upper plasmid band and the linear chromosomal DNA (Figure 9). The lower molecular weight band is probably masked by this smeared region in most of the preparations.

It was obviously desirable to make preparations which eliminated the smeared region. Experiments were carried out to examine the effect of the length of time of the heat treatment on the smeared region. Plate 29 and Figure 10 illustrate the effect of heat treatment time on *Rhizobium meliloti* U45. The lysis temperature was 62°C and the 0 heat treatment step was carried out by lysing at room temperature. Good plasmid bands were observed with the 0 and 2.5 minute heat treatments. A faint plasmid band was observed in the 5 minute treatment. No plasmid bands were visualised in preparations heat treated for longer than 5 minutes.

PLATE 28: A comparison of the lysis by the Kado method of various volumes of strain NZP 5459.

Tracks 1-8 are as in Figure 9 below.

FIGURE 9: A comparison of the lysis by the Kado method of various volumes of strain NZP 5459.



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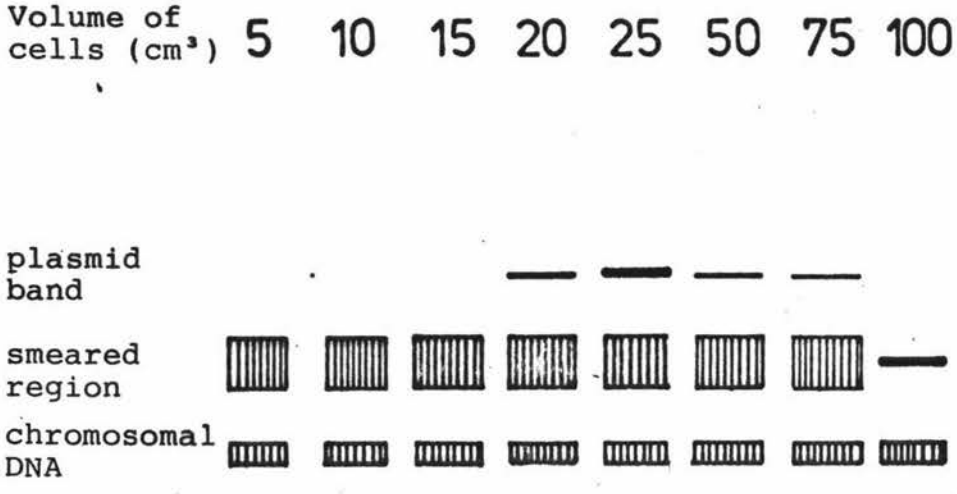

Volume of cells (cm³)
5
10
15
20
25
50
75
100
plasmid band
smeared region
chromosomal DNA

9

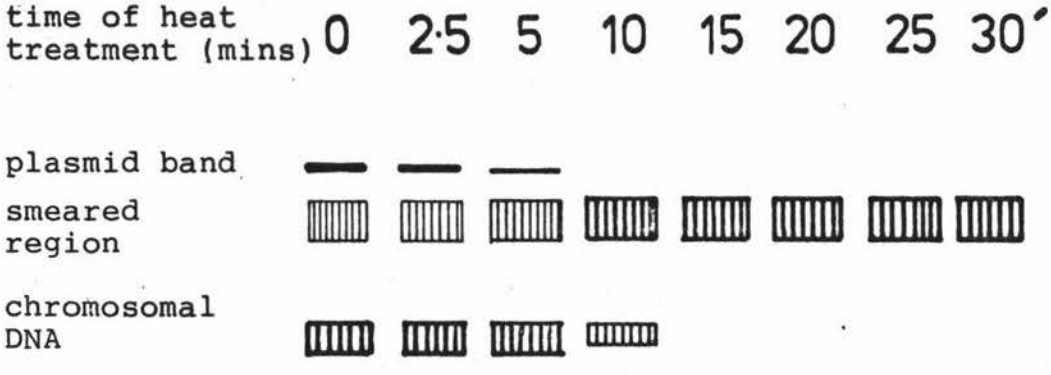
PLATE 29: The effect of heat treatment time on plasmid yield.

Track 1	62°C, 30 min	Track 5	62°C, 10 min
Track 2	62°C, 25 min	Track 6	62°C, 5 min
Track 3	62°C, 20 min	Track 7	62°C, 2.5min
Track 4	62°C, 15 min	Track 8	no heat treatment

FIGURE 10: The effect of heat treatment time on plasmid yield.

(Note. Tracks in diagram are in reverse order to plate 29 above)

29


time of heat treatment (mins)
0
2·5
5
10
15
20
25
30′
plasmid band
smeared region
chromosomal DNA

10

The results show that increasing the heat treatment leads to a decrease in the chromosomal DNA and an increase in the smeared region. Maximum plasmid yield and maximum chromosomal DNA occurred with the same heat treatment. Heat treatment for a sufficient length of time to remove chromosomal DNA also removes all the plasmid DNA from the preparation. Similar results were obtained with strain NZP 5459.

5.52 EFFECT OF TEMPERATURE TREATMENT: Experiments were carried out to determine the effect of various temperatures on the yield of plasmid DNA. Plate 30 and Figure 11 show the effect of various temperature/time combinations on strain NZP 5492.

The best plasmid DNA isolation is at the lowest temperature although this treatment also gives the most chromosomal DNA. The 95°C treatment selectively decreases the yield of the largest plasmid. Increasing the temperature treatment increases the smeared region between the plasmid bands and the chromosomal DNA. This is further illustrated for strains C58 and U45 and a wider range of temperature treatments in Plates 31 and 32. These photographs show that as the temperature of the heat treatment increases the amount of chromosomal DNA in the preparation decreases. However, the yield of the largest plasmid also decreases and the smeared region between the chromosomal and plasmid bands increases as the treatment temperature is increased. The effect of temperature is strain dependent as can be seen from Plate 33 which shows preparations at various temperatures for strain NZP 5492.

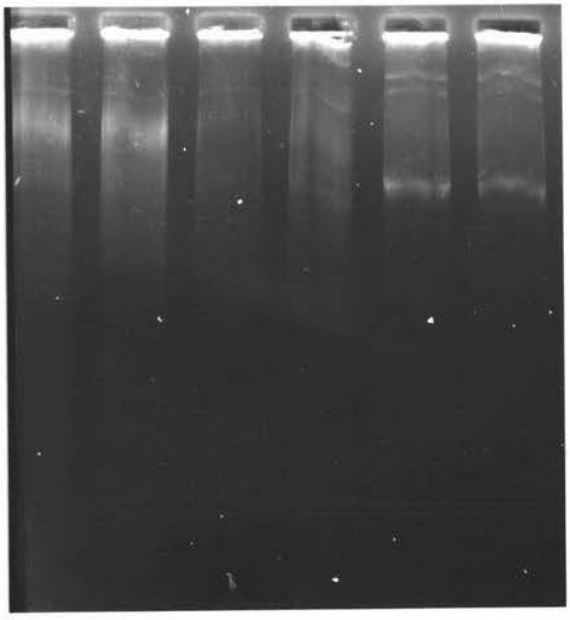
Since the effect of the temperature treatment varied between strains, experiments were carried out to determine the optimum temperature treatment for each strain. The results obtained are summarised in table XVIII. The optimum temperature treatment was considered to be that which gave the most plasmid DNA with the least possible chromosomal DNA.

PLATE 30: The effect of various temperature/time combinations on strain NZP 5492.

Tracks 1 and 2 95°C for 5 mins
Tracks 3 and 4 80°C for 10 mins
Tracks 5 and 6 62°C for 20 mins

FIGURE 11: The effect of various temperature/time combinations on strain NZP 5492.

(Note. Tracks in diagram are in reverse order to plate 30 above)



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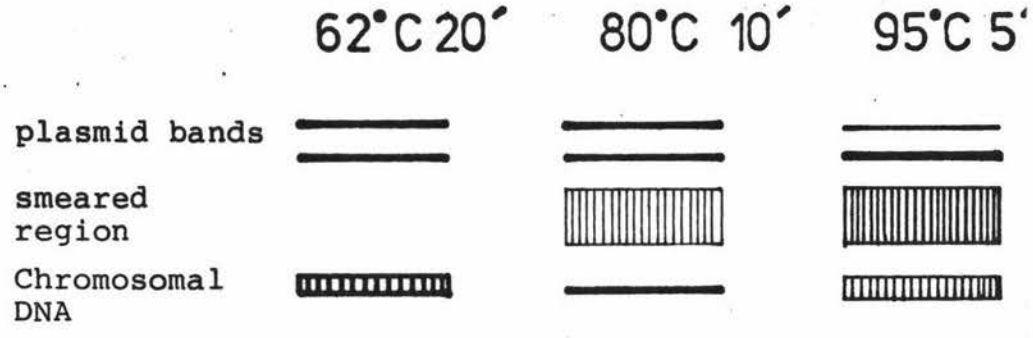

62°C 20′
80°C 10′
95°C 5′
plasmid bands
smeared region
Chromosomal DNA

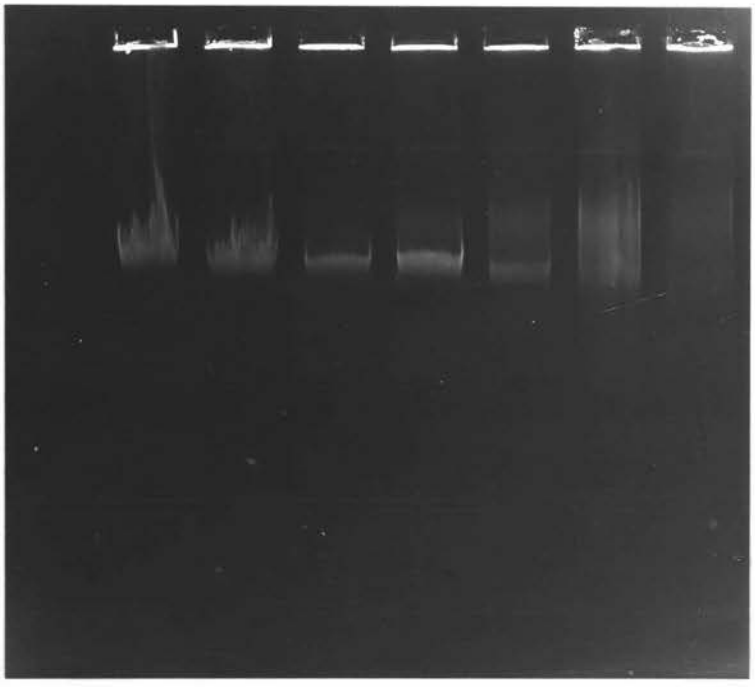
11

PLATES 31 & 32: The effect of various temperatures on the yield of plasmid DNA by the Kado method.

Temperature treatments were carried out for 30 mins. Left to right: ice; room temp; 30°C; 37°C; 45°C; 50°C; 55°C; 65°C.
The 0.7% gels were electrophoresed for $4\frac{1}{2}$ hours at 110V.

(a) Plate 31: *Agrobacterium tumefaciens* C58

(b) Plate 32: *Rhizobium meliloti* U45



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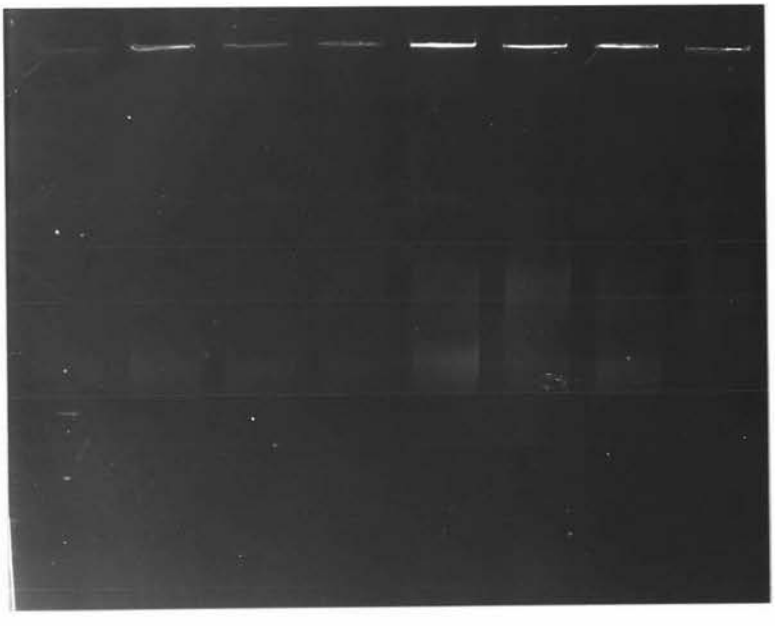

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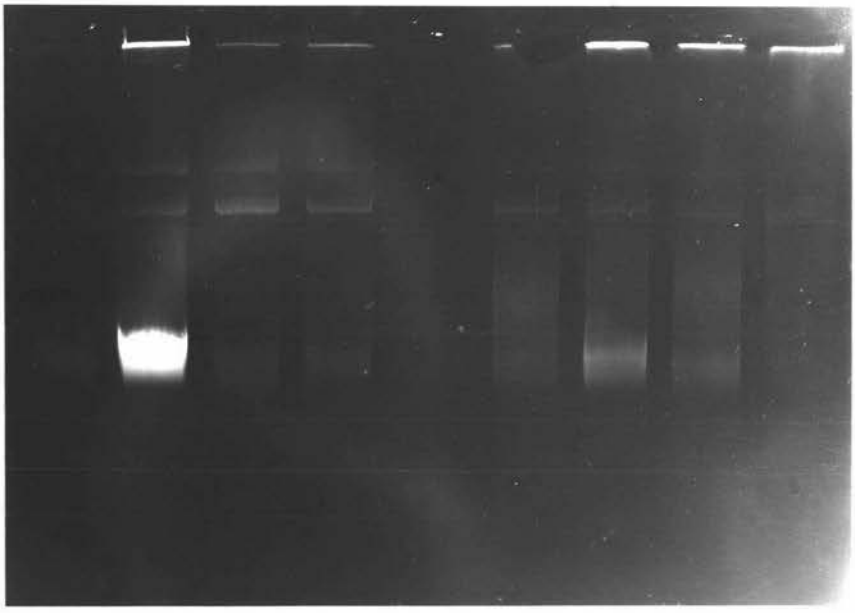
PLATE 33: The effect of temperature treatment on strain NZP 5492.

Track 1	ice	Track 6	45°C
Track 2	room temp	Track 7	50°C
Track 3	30°C	Track 8	55°C
Track 4	37°C	Track 9	65°C
Track 5	empty		

The 0.7% gel was electrophoresed for 5 hours at 110V



33

TABLE XVIII: Optimum temperature treatments with Kado Method for *Rhizobium phaseoli* strains

Strain	Optimum temperature treatment	Number of plasmid bands obtained
NZP 5459	room temp —— 37°C	1
NZP 5260	55°C	3
NZP 5462	room temperature	2
NZP 5097	room temperature	2
NZP 5492	room temp —— 55°C	2

5.53; EFFECT OF SODIUM CHLORIDE: It was suggested (D.B. Scott, personal communication) that 0.5M NaCl added to the lysate before extraction with phenol may remove chromosomal DNA. Experiments to study the effect of 0.5M NaCl at various temperatures were carried out on strains NZP 5097, 5260, 5492 and 5462. Strain NZP 5097 (Plate 34) Plasmid bands can be seen for all temperatures without the salt treatment. Chromosomal DNA is greatly reduced in the 55°C and 65°C treatments (Tracks 5 and 7). Salt treatment results in a considerable loss of plasmid yield (Tracks 2, 4, 6 and 8).

Strain NZP 5260 (Plate 35). The best plasmid isolation was with a 55°C temperature treatment without salt (Track 6). Plasmid yield is reduced with all other temperature treatments and with the salt treatment at all temperatures.

Strain NZP 5492 (Plate 36). The plate shows successful plasmid extractions from a wide range of heat treatments without salt (Tracks 3, 5 and 7). Salt treatment reduces both the plasmid and chromosomal DNA yield (Tracks 2, 4, 6 and 8).

Strain NZP 5462 (Plate 37). The conditions in Track 3 (room temperature, no salt) are optimum for the isolation of plasmids from this strain. The smeared region increases

PLATE 34: The effect of 0.5M NaCl at various temperatures on strain NZP 5097.

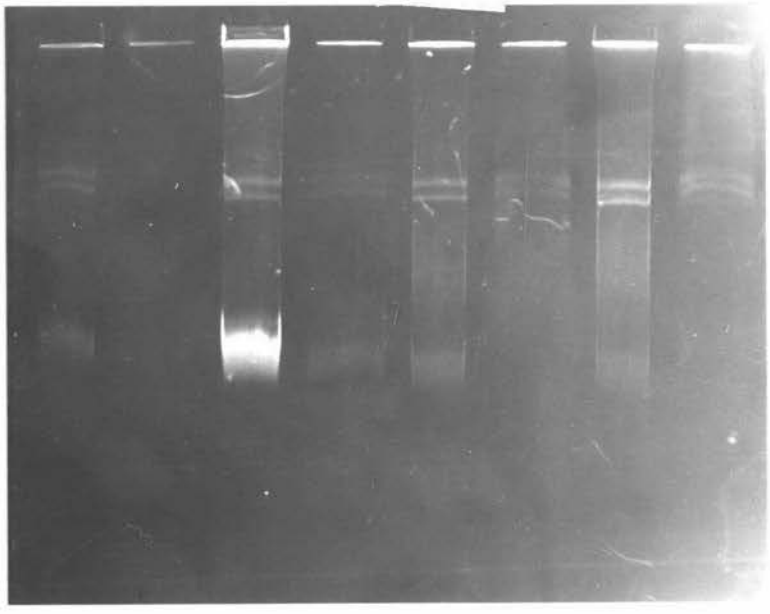
Track 1 ice, no salt
Track 2 ice + salt
Track 3 room temp, no salt
Track 4 room temp + salt
Track 5 55°C, no salt
Track 6 55°C + salt
Track 7 65°C, no salt
Track 8 65°C + salt

The 0.7% agarose gel was electrophoresed for 5 hours at 110 V.

PLATE 35: The effect of 0.5M NaCl at various temperatures on strain NZP 5260

Track 1 ice, no salt
Track 2 ice + salt
Track 3 room temp, no salt
Track 4 room temp + salt
Track 5 empty
Track 6 55°C, no salt
Track 7 55°C + salt
Track 8 65°C, no salt
Track 9 65°C + salt

The 0.7% agarose gel was electrophoresed for 6 hours at 110V



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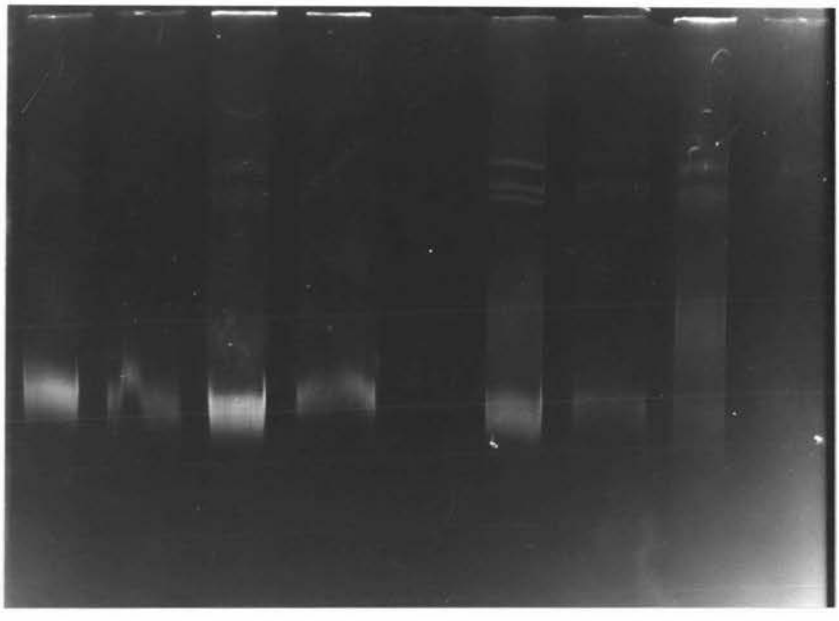


35

PLATE 36: The effect of 0.5M NaCl at various temperatures on strain NZP 5492

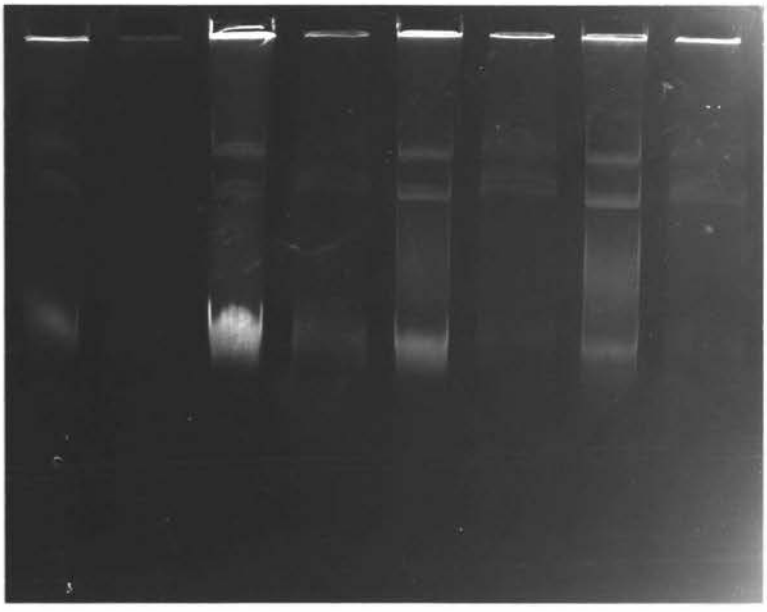
Track 1 ice, no salt
Track 2 ice + salt
Track 3 room temp, no salt
Track 4 room temp + salt
Track 5 55°C, no salt
Track 6 55°C + salt
Track 7 65°C, no salt
Track 8 65°C + salt

The 0.7% agarose gel was electrophoresed for 5 hours at 110V

PLATE 37: The effect of 0.5M NaCl at various temperatures on strain NZP 5462

Track 1 ice, no salt
Track 2 ice + salt
Track 3 room temp, no salt
Track 4 room temp + salt
Track 5 55°C, no salt
Track 6 55°C + salt
Track 7 65°C, no salt
Track 8 65°C + salt

The 0.7% agarose gel was electrophoresed for 5 hours at 110V



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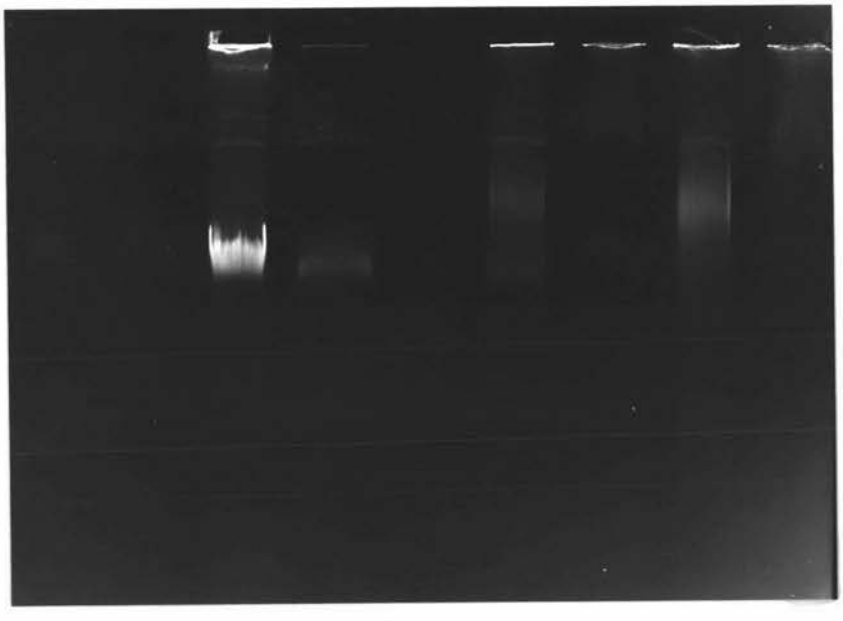


37

in higher temperature treatments (Tracks 6 and 8) and the upper plasmid is selectively lost. Salt treatment results in little chromosomal DNA in the preparation (Tracks 2, 4, 7 and 9) but plasmid yield is also reduced. The higher molecular weight plasmid is not present in any of the salt treated preparations.

We did not consider salt treatment any use because it does not permit sufficiently high plasmid yields even though it does reduce the amount of chromosomal DNA in the preparation.

5.54 EFFECT OF LYSING SOLUTION pH: Inability to maintain a reproducible pH in the lysing solution was postulated as a reason for variations in plasmid yield from a given strain under the same conditions of temperature and cell density.

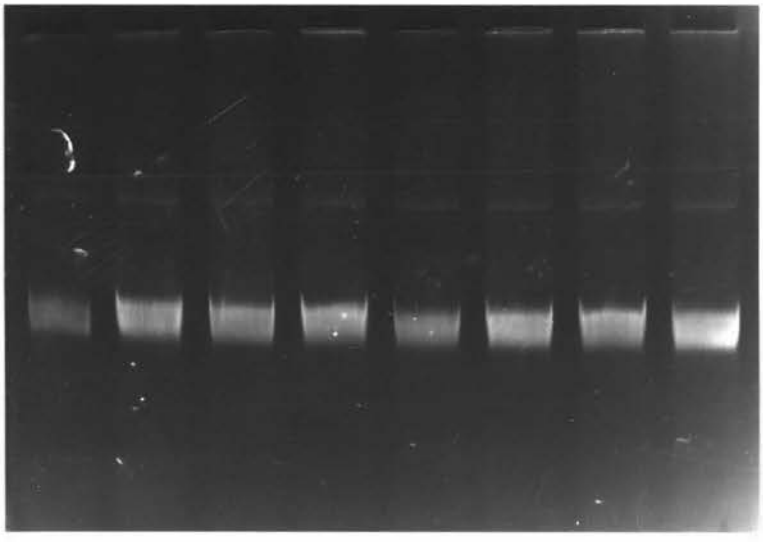
Kado's paper describes the adjustment of 100cm³ of lysing buffer to pH 12.6 by the addition of 1.6cm³ of 2M NaOH. It is difficult to accurately measure pH in this region. A range of lysing solutions was therefore prepared by adding various volumes of 2M NaOH to the lysing buffer. Table XVIV gives the details of the lysing solutions used to lyse strain NZP 5492.

A culture of NZP 5492 (OD_{600} 5.5) was lysed with each of the lysing solutions in Table XVIV. Heat treatment was at 55°C for 30 minutes. There was no difference in plasmid yield over the pH range (Plate 38). Further experiments with other strains (data not shown) confirmed that the pH was not as critical as expected.

An experiment was carried out to examine whether high pH treatments could be used to eliminate chromosomal DNA. Strain NZP 5492 was lysed over a range of lysing solutions from 1.5cm³ 2M NaOH per 100cm³ to 5.0cm³ 2M NaOH per 100cm³ lysing solution. Chromosomal DNA was never completely

PLATE 38: The effect of lysing solution pH on the isolation of plasmid from strain NZP 5492 by the Kado method:

The pH of the lysing solution in tracks from left to right corresponds to Table XVIV from top to bottom.



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eliminated although it was reduced in the three highest pH treatments. A smeared area, possibly from degredation of the higher molecular weight plasmid band was present in all treatments above 2.0cm³ 2M NaOH per 100cm³ lysing solution.

TABLE XVIV: Lysing solutions used for Kado Method:

Volume of 2M NaOH added to 100cm³ lysing solution	pH reading (not corrected)	Na^+ ion pH Correction	Corrected pH
1.0	11.65	0.08	11.7
1.3	11.85	0.09	11.9
1.4	12.0	0.11	12.1
1.5	12.2	0.12	12.3
1.6	12.3	0.15	12.45
1.7	12.35	0.15	12.5
1.8	12.4	0.16	12.6
2.0	12.45	0.18	12.65

5.55 CONCLUSIONS FROM KADO METHOD: We concluded that the important factors affecting the Kado method are strain, number of cells and temperature of the heat treatment. pH of the lysing solution was not a critical factor.

0.5M NaCl treatment removed large plasmids as well as chromosomal DNA. Chromosomal DNA could not be eliminated by high pH lysing solutions (pH 13.0). We were unable to find conditions which discriminated sufficiently between large plasmids and chromosomal DNA. Consequently it was not possible to use this method to obtain plasmid preparations free of chromosomal DNA for restriction enzyme analysis and it was therefore necessary to separate the plasmid and chromosomal DNA obtained from the extraction method.

5.6 General Conclusions from Plasmid Extraction Methods:

Large plasmids were demonstrated in *Rhizobium phaseoli* strains. The number of plasmids varied from 1 to 3 per strain. The same number of plasmids were not always isolated with a particular method or from the same strain with different methods (Table XX). The variable isolation of a third plasmid band for strain NZP 5492 by some methods indicates that this large plasmid is present but difficult to isolate by these methods. The Alkaline lysis method, Anderson's method, Eckhardt method and Casse method were used only to verify the presence of large plasmids in the *Rhizobium* strains being examined. Since these methods could not provide purified plasmid in sufficient quantity for later analysis they were not fully examined.

The methods of Currier and Nester (1976) and Koekman (1980) were used to produce large quantities of plasmid preparation of all strains for later purification by gradient centrifugation (Method 6). In most cases these methods gave the same plasmid bands as the screening methods above. The exception was strain NZP 5462 which had 2 plasmid bands with the Eckhardt method but only 1 with the Currier and Nester method. Ekhardts method is considered particularly suitable for the isolation of very large molecular weight plasmids which are probably broken up by the shearing and alkali denaturation steps in the Currier and Nester method.

The method of Kado & Liu (1979) was examined in considerable detail because of the possibility of producing reasonably large quantities of plasmid sufficiently pure for restriction enzyme analysis (see Kado Conclusions, section 5.55).

After the majority of the experimental work for this thesis was completed, the method of Schwinghamer (1980) was obtained. This method incorporates a detergent wash - osmotic shock method to lyse the bacteria. Schwinghamer considers that

TABLE XX: Plasmid bands obtained by various methods

Method \ Strain NZP	Homology Group 1							Homology Group 2				
	5097	5232	5260	5492	5459	5547	5479	5384	5456	5443	5462	5463
Alkaline lysis	2	0	3	1	0	-	-	0	0	0	0	0
Andersen	2	2	3	2	1	-	-	0	0	1	1	0
Eckhardt	-	-	-	2	-	-	-	-	-	-	2	-
Casse	-	-	-	2 or 3 v	1	-	-	-	-	-	-	-
Currier and Nester	2	-	3	2	1	2	2	-	-	-	1	-
Koekman	2	-	-	2	1	2	2	-	-	-	-	-
Kado	2	1	-	2 or 3 v	1	2	2	1?	0	2	1	0

o = no bands obtained by the method
- = did not perform method on strain
v = variable number of bands obtained

Note: - strains which did not give bands initially with a method but after modification of a method gave plasmid bands are not considered variable.

this is more gentle on the supercoiled plasmid and enables the isolation of larger plasmids than the method of Currier and Nester.

We have performed this method (Section 5.93) on strain NZP 5492 and obtained 2 plasmid bands which was the same result as with the other methods used.

6. PURIFICATION OF PLASMID DNA BY GRADIENT CENTRIFUGATION

Since no plasmid extraction method was able to give sufficient quantities of plasmid DNA free of chromosomal DNA for restriction enzyme analysis it was necessary to separate the chromosomal and plasmid DNA in the crude extract. Sucrose gradients were tried first because of their relative cheapness and speed compared with cesium chloride gradients.

6.1 Sucrose Gradients

A 5-20% linear sucrose gradient was prepared in a Beckman SW 41 centrifuge tube (Method 6.1). Strain NZP 5492 DNA solution, $1cm^3$ of a $2mg/cm^3$ solution containing a mixture of two plasmids and chromosomal DNA was layered onto the gradient. The gradient was centrifuged at 40,000rpm for 45 minutes. Fractions ($0.3cm^3$) were collected from the top of the tube with an Isco fractionator (Method 6.13). The majority of the lower molecular weight plasmid DNA was contained in fractions 9 and 10 while the higher molecular weight plasmid was contained in fractions 14 to 17 inclusive. Chromosomal DNA was present in all fractions. A longer centrifugation for 120 minutes failed to improve the separation between plasmid and chromosomal DNA. We concluded that a 5-20% sucrose gradient was insufficient to separate plasmid and chromosomal DNA and decided to use a steeper gradient.

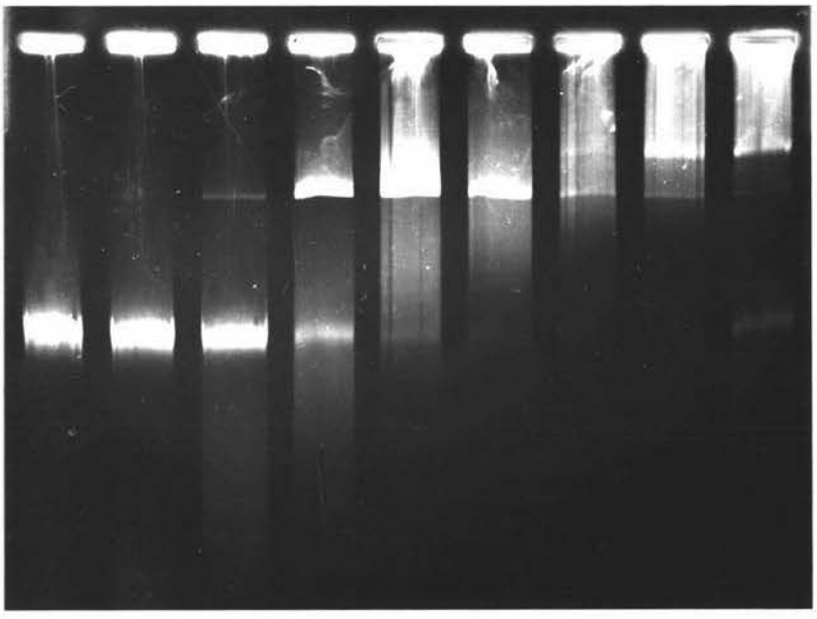
Plates 39 and 40 show fractions from a 10-30% linear sucrose gradient after centrifugation at 40,000rpm for 40 mins. The figure shows chromosomal DNA in all fractions, however the main chromosomal DNA is in fractions 2-4. The lower molecular weight plasmid is concentrated in fractions 5-7 and the higher molecular weight plasmid in tubes 9-12. Fractions 9-12 also contain traces of the lower molecular weight plasmid.

PLATES 39 and 40: Fractions from a 10-30% sucrose gradient.

Plate 39: Fractions 2-10

Plate 40: Fractions 11-16, 26, 38 and 39 (bottom of tube)

The 0.7% agarose gels were electrophoresed at 110V for 5 hours.



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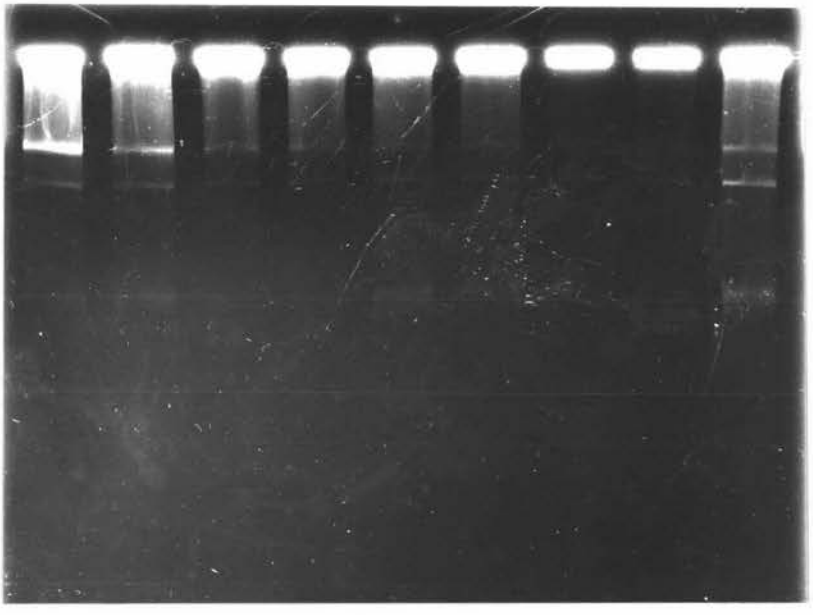

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We concluded from the failure of the gradient to separate the DNA that the gradient was overloaded. A longer time for the centrifugation would also have given better separation.

The DNA solution was therefore dilued to 0.4mg per cm^3 and 1.0 cm^3 was layered on to a 10-30% sucrose gradient and centrifuged at 40,000rpm for 120 minutes. Most of the chromosomal DNA was in fractions 6 to 17 and the plasmid was in fractions 18-20. However, there was still some chromosomal DNA in the plasmid fractions. A longer spin of 200 minutes under the same conditions increased the separation of the chromosomal and plasmid DNA.

Plates 41 and 42 show the chromosomal DNA in fractions 8-14 and plasmid DNA substantially free of chromosomal DNA in fractions 23 and 24.

We were unable to find conditions that would separate plasmid and chromosomal DNA when using the undiluted DNA (2.0mg per cm^3). Consequently we could not use sucrose gradients to obtain sufficient purified plasmid DNA for restriction enzyme digestion.

6.2 Cesium Chloride Gradients:

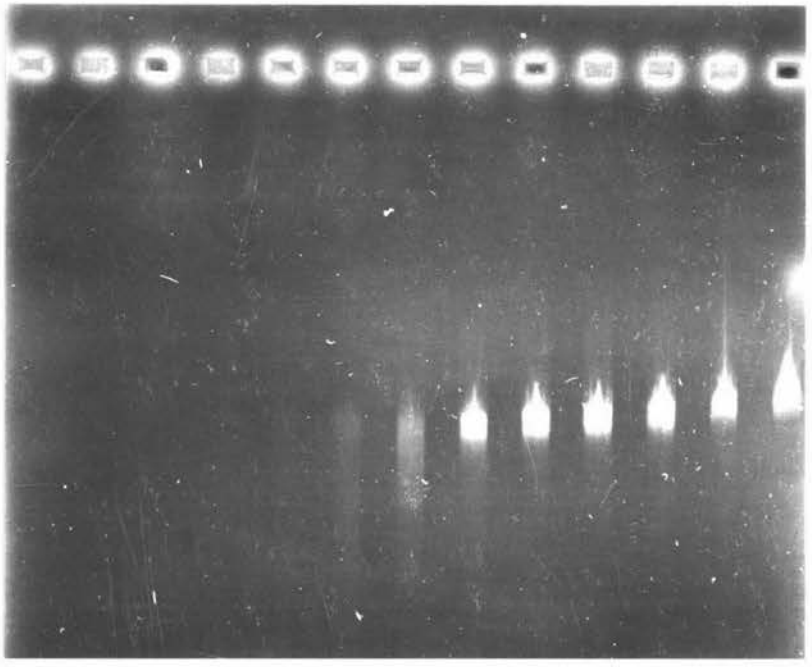
Plasmid preparations from the Currier and Nester and Koekman methods (Methods 5.91 and 5.92) were mixed with cesium chloride (1gm CsCl in 1cm^3 plasmid extract) to give a starting density of 1.5825gm/cm^3 and centrifuged in a Beckman 50Ti rotor at 45,000rpm for 48 hours to obtain a linear gradient from 1.42g - 1.82g / cm^3. The refractive index of 5 drop fractions from this gradient was measured (Figure 12). The plasmid DNA had a bouyant density of approximately 1.6g/cm^3 (Refractive index 1.39). The bands were visualised under u.v. irradiation (Method 6.24), a typical result is shown in Plate 43. The DNA was recovered from these two bands (Method 6.24) and electrophoresed on a 0.7% agarose gel (Method 5.5) to verify the separation

PLATES 41 AND 42: Fractions from a 10-30% sucrose gradient centrifuged for 200 mins at 40,000rpm.

Plate 41: Fractions 1-13

Plate 42: Fractions 14-26

The 0.7% agarose gels were electrophoresed for 5 hours at 110V



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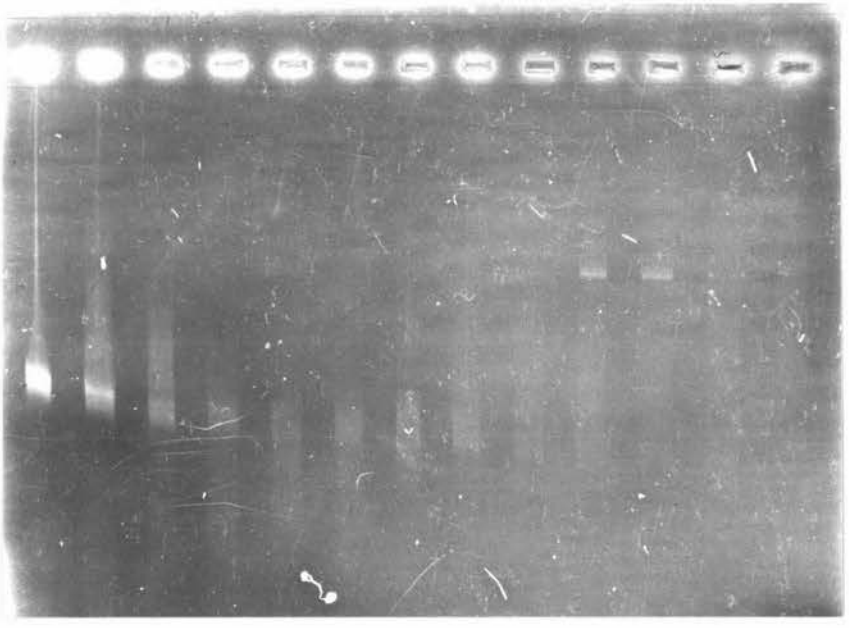

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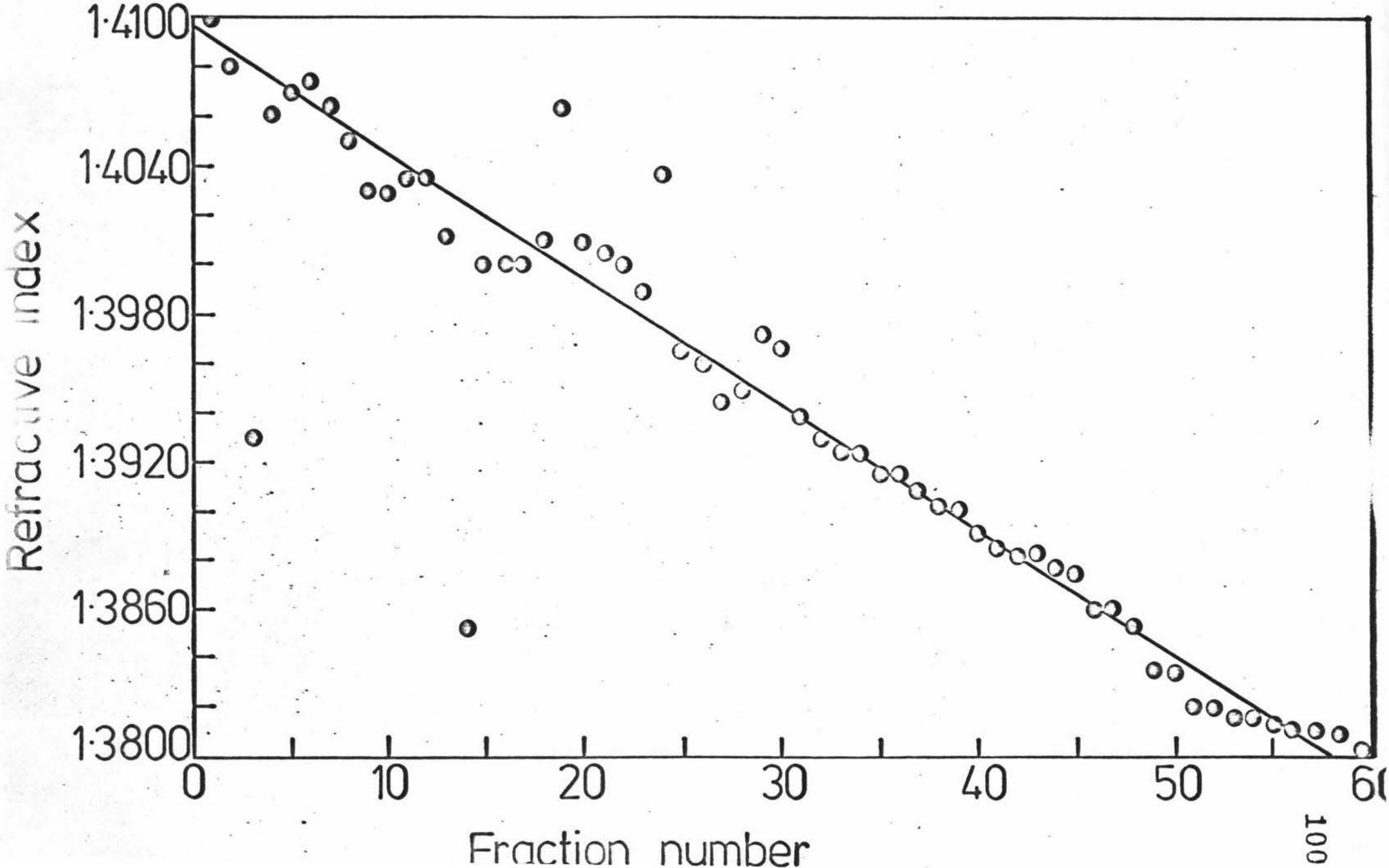
FIGURE 12: Cesium chloride gradient formed in a Beckman 50Ti rotor at 45,000rpm for 48 hours (solid line represents the theoretical gradient)


1·4100
1·4040
1·3980
1·3920
1·3860
1·3800
Refractive index
0
10
20
30
40
50
60
Fraction number
100

of plasmid and chromosomal DNA. Plate 44 shows that plasmid DNA only was recovered from the lower band and linear chromosomal DNA from the upper band. Therefore cesium chloride density gradients under the conditions described are adequate for separating plasmid from chromosomal DNA and were used to obtain purified plasmid for restriction endonuclease digestion from a number of *R. phaseoli* strains.

7. ESTIMATION OF THE MOLECULAR WEIGHTS OF WHOLE PLASMIDS

The molecular weights of the *R. phaseoli* plasmids were estimated by comparing their relative mobility to that of plasmids of known molecular weight. Plate 45 shows a gel containing reference plasmids from *Escherichia coli*, *Rhizobium meliloti* and *Agrobacterium tumefaciens*, and *Rhizobium phaseoli* plasmids of unknown size. Figure 13 shows the plasmid bands from this photograph. The relative mobilities of the bands were measured from the Figure (Tables XXI, XXII).

Log_{10} molecular weight of the reference plasmids was plotted against log_{10} relative mobility to produce a standard curve (Figure 14). The molecular weight of the *R. phaseoli* plasmids (Table XXII estimated from their relative mobility.

The *R. phaseoli* plasmids ranged in size from 66 mega daltons to 316 megadaltons. The larger molecular weight plasmids especially the 288mD plasmid of NZP 5479 and the 316mD plasmid of NZP 5462 were not found in every plasmid isolation from these strains. Because of their large size they are more susceptible to damage and loss during the isolation procedures.

PLATE 43: Cesium chloride gradient visualised under u.v. illumination. Gradient was centrifuged for 48 hours at 45,000 rpm in a Beckman 50Ti rotor

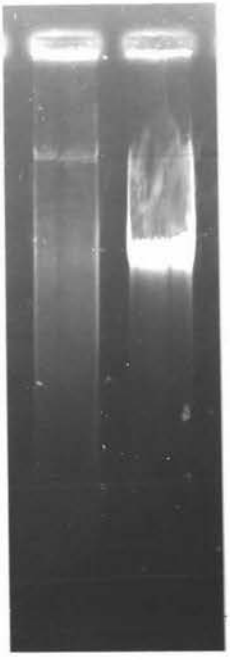
ch - linear chromosomal DNA band
p - supercoiled plasmid band

PLATE 44: DNA recovered from cesium chloride density gradient

Track 1 plasmid DNA from the lower band

Track 2 linear chromosomal DNA from the upper band

43



44

PLATE 45: Reference plasmids of known molecular weight and *Rhizobium phaseoli* plasmids of unknown molecular weight.

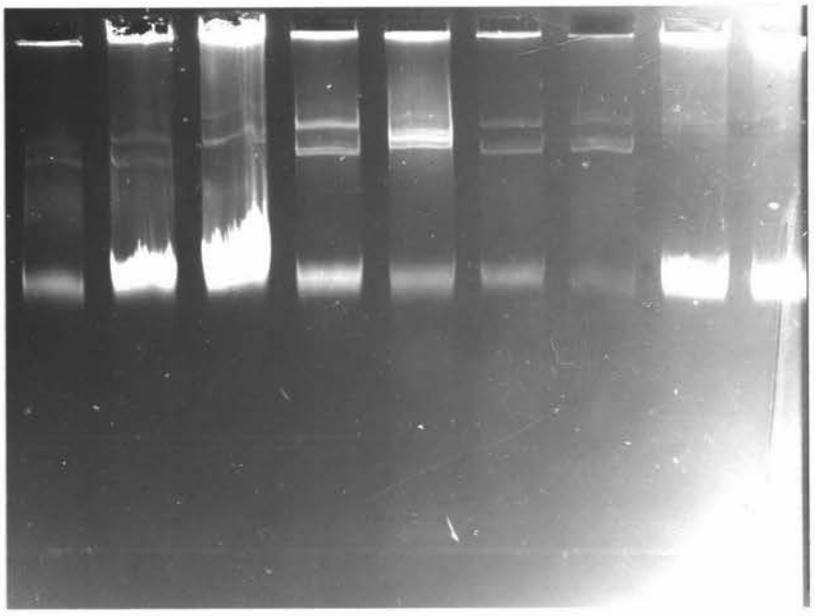
Track 1 *Rhizobium meliloti* U45 (101mD) and *E. coli* R100-1 (60mD)

Track 2 *Agrobacterium tumefaciens* C58 ((a) 130mD, (b) 275mD) and *E. coli* R100-1 (60mD)

Track 3	*Rhizobium sp.*	NZP 5462
Track 4	*R. phaseoli*	NZP 5492
Track 5	*R. phaseoli*	NZP 5097
Track 6	*R. phaseoli*	NZP 5492 (B5/8)
Track 7	*R. phaseoli*	NZP 5492 (B5/1)
Track 8	*R. phaseoli*	NZP 5479
Track 9	*R. phaseoli*	NZP 5547

The 0.7% agarose gel was electrophoresed for 5 hours at 110V

FIGURE 13: Plasmid bands from plate 45



45

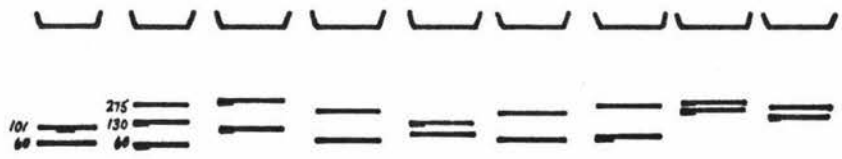


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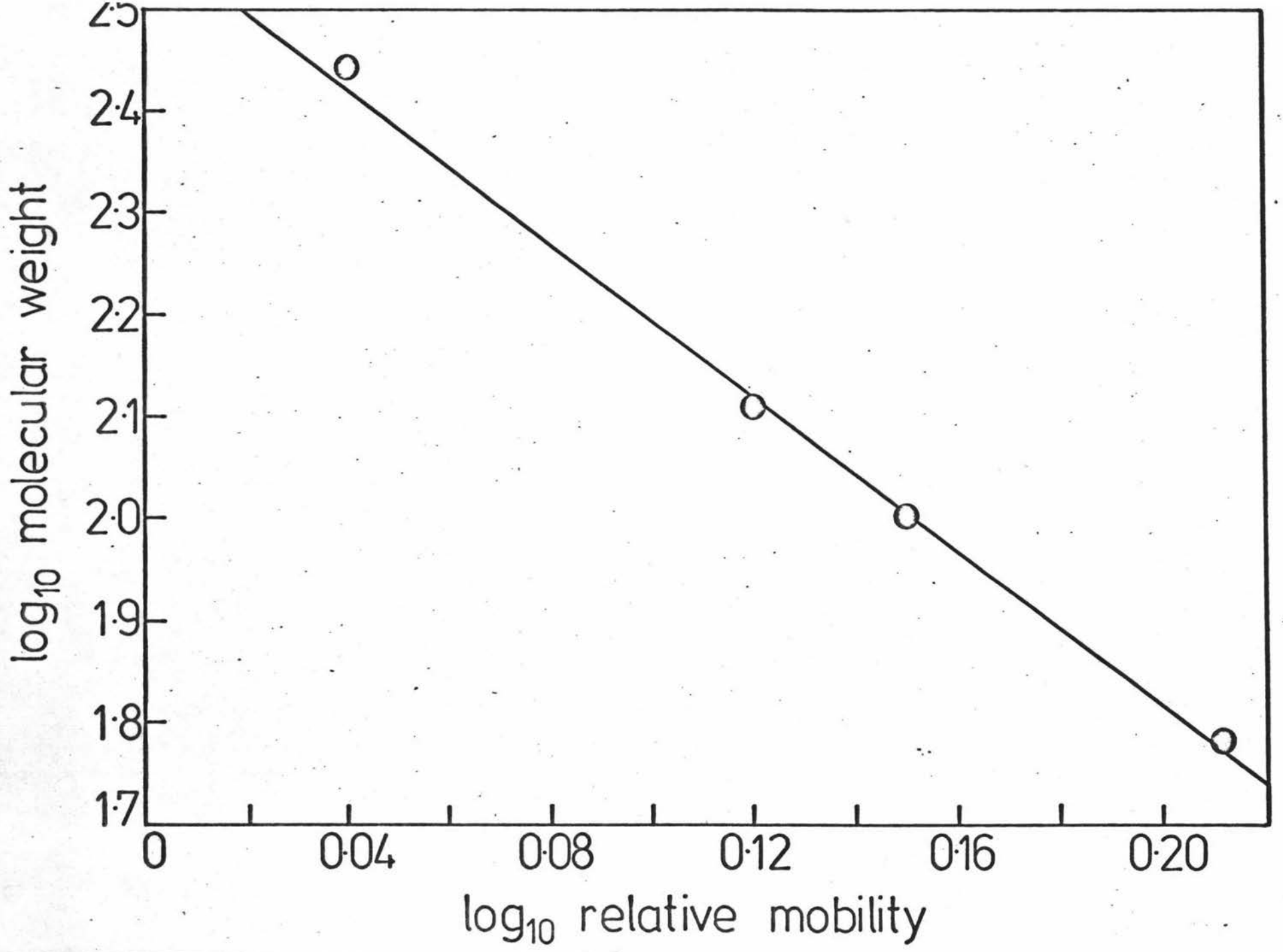
TABLE XXI: Relative mobility of plasmids of known molecular weight

Plasmid		Plasmid molecular weight (Md)	$\log_{10}$ plasmid molecular weight	Relative mobility	$\log_{10}$ relative mobility
E. *coli*	R100-1	60	1.78	1.63	0.21
R. *meliloti*	U45	101	2.00	1.40	0.15
A. *tumefaciens*	C-58(a)	130	2.11	1.33	0.12
A. *tumefaciens*	C-58(b)	275	2.44	1.10	0.04

TABLE XXII: Molecular weight of *R. phaesoli* plasmids estimated from their relative mobility

	Relative mobility	$\log_{10}$ relative mobility	$\log_{10}$ estimated molecular weight	Estimated molecular weight (Md)
NZP 5462 (a)	1.05	0.02	2.50	316
NZP 5462 (b)	1.43	0.16	1.97	93
NZP 5492 (a)	1.20	0.08	2.27	186
NZP 5492 (b)	1.57	0.20	1.82	66
NZP 5097 (a)	1.36	0.13	2.08	120
NZP 5097 (b)	1.49	0.17	1.93	85
NZP 5479 (a)	1.08	0.03	2.46	288
NZP 5479 (b)	1.21	0.08	2.27	186

FIGURE 14: Standard curve relating $\log_{10}$ relative mobility to $\log_{10}$ molecular weight for reference plasmids of known molecular weight (plate 45)


log10 molecular weight
2·5
2·4
2·3
2·2
2·1
2·0
1·9
1·8
1·7
0
0·04
0·08
0·12
0·16
0·20
log10 relative mobility

8. RESTRICTION ENDONUCLEASE DIGESTS

The purified whole plasmids obtained from cesium chloride gradients (method Section 6.2) were digested with restriction endonucleases as desribed in Methods Section 7.

Plate 46 shows a digest of *Rhizobium phaseoli* strain NZP 5492 from DNA homology group 1 with *Eco* RI, *Hin*dIII and *Bam* HI. In each case the fragments can be compared with the digest of lambda (λ) with the same restriction enzymes. Also included on this gel is an *Eco* RI digest of *Rhizobium* sp. NZP 5462 from DNA homology group 2.

The fragment pattern obtained on this gel was transferred to paper using vernier callipers to facilitate comparison of the bands (Figure 15). The relative mobilities of the digest fragments from lambda were used to construct a standard curve (Appendix Figure A1) relating $\log_{10}$ relative mobility to $\log_{10}$ molecular weight of the fragments (Appendix Table A1). The molecular weights of the unknown digest fragments were determined from this standard curve.

The fragment sizes and mobilities for the plasmid from *R. phaseoli* NZP 5492 digested with *Eco* RI, *Bam* HI and *Hin*dIII are given in appendix Table AII. *Eco* RI digestion gave 15 fragments adding up to a total molecular weight of 56.4Md, *Bam* HI and *Hin*dIII digestion both produced 19 fragments adding up to total molecular weights of 77.3Md and 55.4Md respectively. The dissimilarities in the total molecular weight are probably due to the fact that if two fragments are of identical size they only form one band and are only counted once in determining the total molecular weight.

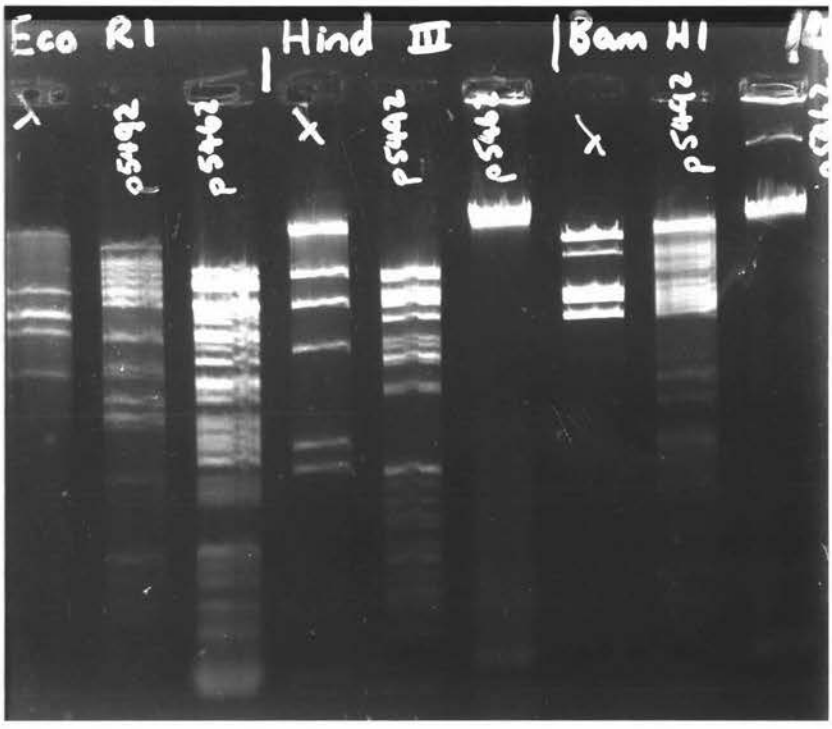
Eco RI digestion of the plasmid from *Rhizobium* sp. NZP 5462 (from DNA homology group 2) gave 23 fragments with

PLATE 46: Restriction enzyme digests of *R. phaseoli* NZP 5492 and NZP 5462

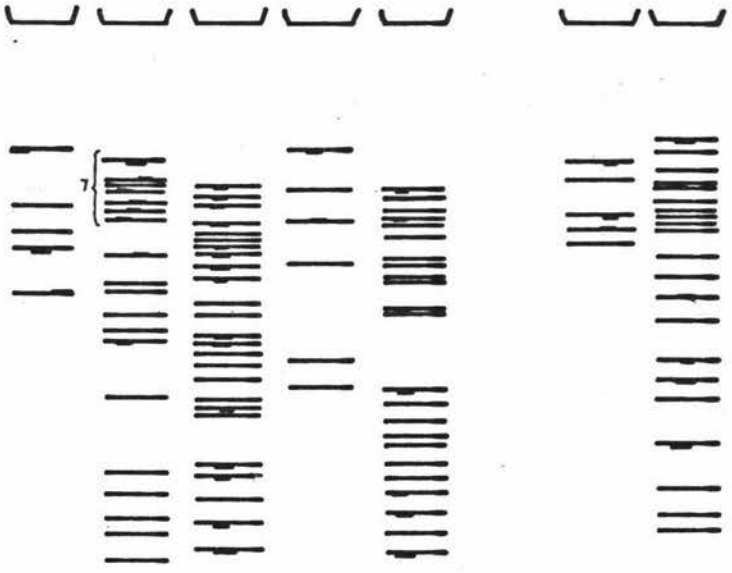
Track 1 *Eco* RI digest of phage λ
Track 2 *Eco* RI digest of NZP 5492
Track 3 *Eco* RI digest of NZP 5462
Track 4 *Hin*dIII digest of phage λ
Track 5 *Hin*dIII digest of NZP 5492
Track 7 *Bam* HI digest of phage λ
Track 8 *Bam* HI digest of NZP 5492

Electrophoresis was for 2.75 hours at 110V in a 1% gel.

FIGURE 15: Bands from plate 46 (Restriction enzyme digests of *R. phaseoli* NZP 5492and NZP 5462)



46



15

a total molecular weight of 61.5Md. The overall fragment pattern was different from that obtained for NZP 5492 (DNA homology group 1) although some bands were of identical size.

Plate 47 and Figure 16 and plate 48 and Figure 17 show two different *Eco* RI digests of the standard lambda, *R. phaseoli* strains NZP 5492 and NZP 5097 from DNA homology group 1 and *Rhizobium* sp. NZP 5462 from DNA homology group 2. Standard curves were prepared as before for both digests (Appendix Figure A2 and A3) from the molecular weights and relative mobilities of lambda (Appendix, Tables AIV and AVI). The numbers of fragments and estimated molecular weight of the fragments and plasmids are given in Appendix Tables AV and AVII. It can be seen from the data and the plates that the DNA homology group 1 strains NZP 5492 and NZP 5097 are not similar in their fragment pattern although there are some fragments of similar molecular size. The number and size of the fragments from NZP 5462 (DNA homology group 2) are different from the numbers and sizes of the fragments from the DNA homology group 1 strains.

The differences between the two digests (Plate 47 & 48) may be due to incomplete digestion of the plasmid in the second digest (Plate 48) resulting in the presence of higher molecular weight bands.

The estimated molecular weights obtained from digest fragments and the estimated molecular weights for the whole plasmids (Section 7) are compared in Table XXIII. It was expected that the estimated molecular weights obtained by adding up the restriction enzyme fragments would be lower than that of the whole plasmids because of the failure to observe and add on small bands and because bands of similar molecular weight would mask each other and only be counted as one band. This was demonstrated in the results (Table XXIII).

PLATE 47: *Eco* RI digests of λ (Track 1) *R. phaseoli* NZP 5492 (Track 2), NZP 5097 (Track 4) and NZP 5462 (Track 5)

Electrophoresis was for 3 hours at 110V in a 0.7% gel

FIGURE 16: Bands from plate 47 (*Eco* RI digests of λ, *R. phaseoli* NZP 5492, NZP 5097 and NZP 5462)

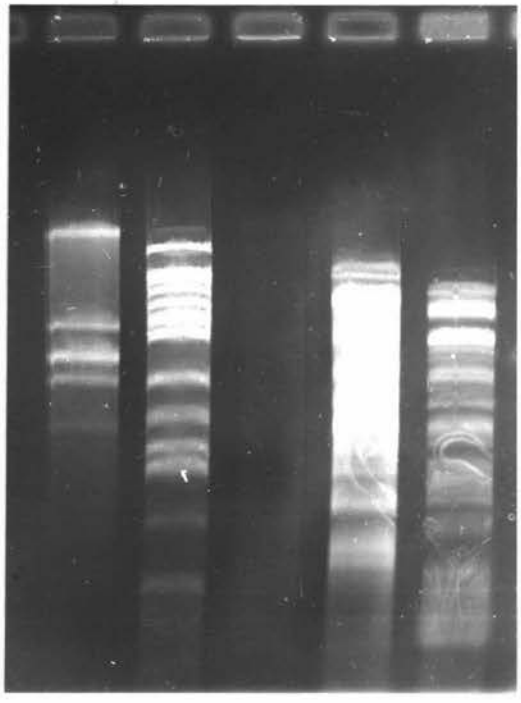


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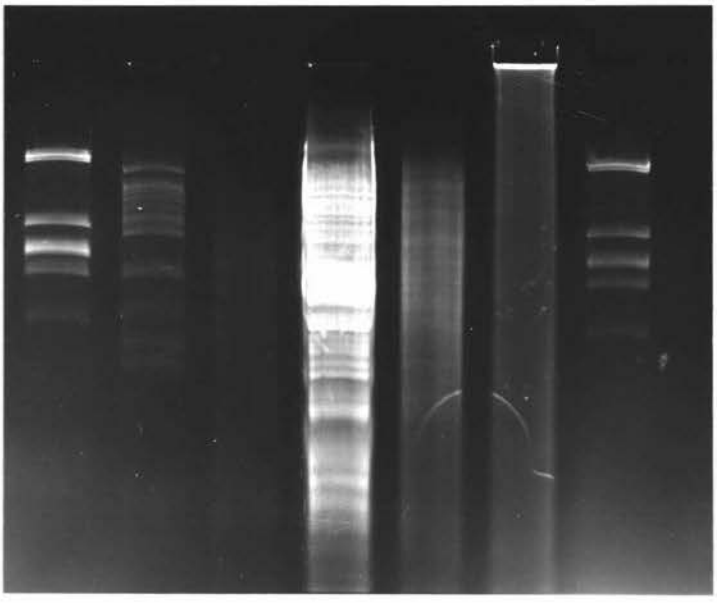
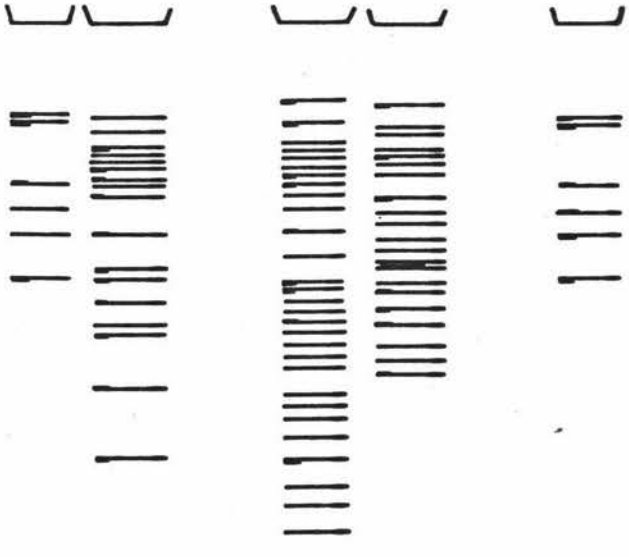
PLATE 48: *Eco* RI digests of λ (Tracks 1 and 7) *Rhizobium phaseoli* NZP 5492 (Track 2), NZP 5097 (Track 4) and NZP 5462 (Track 5)

Electrophoresis was for 6.5 hours at 60V in a 1% gel.

FIGURE 17: Bands from plate 48 (*Eco* RI digests of λ, *R. phaseoli* NZP 5492, NZP 5097 and NZP 5462)



48



17

TABLE XXIII: A comparison between the estimated molecular weights of intact *R. phaseoli* plasmids and molecular weights caclulated from restriction enzyme digests of those plasmids

Rhizobium Strain	Molecular weight (Md)						
	Digest Number [a, b]					Digest Mean	Whole Plasmid
	Eco^{1}RI	*Eco*^{2}RI	*Eco*^{3}RI	*Bam*^{3}HI	*Hin*^{5}dIII		
5492	56.4 (15)	51.6 (13)	76.9 (16)	77.3 (19)	55.4 (19)	64.6	66
5097	-	62.8 (21)	94.5 (23)	-	-	78.6	85
5462	61.5 (23)	65.1 (23)	94.7 (21)	-	-	74	93

(a) Digests 1, 4 and 5 ≡ Plate 46, Figure 15
Digest 2 ≡ Plate 47, Figure 16
Digest 3 ≡ Plate 48, Figure 17

(b) Figures given in brackets indicate total numbers of bands observed in each digest

8.1 Comparison of *R. phaseoli* NZP 5492 B5/8 (effective) and the ineffective mutant NZP 5492 B5/1.

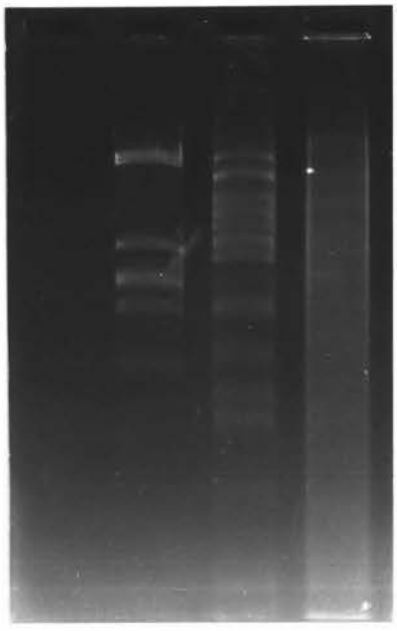
Eco RI digests of the molecular weight standard lambda and plasmids from *R. phaseoli* NZP 5492 B5/8 and NZP 5492 B5/1 are shown in Plate 49 and Figure 18. A standard curve (Appendix Figure A4) was prepared from the molecular weight and relative mobilities of lambda (Appendix Table AVIII). The molecular weights of the plasmid fragments were estimated from this standard curve (Appendix Table IX). Strain NZP 5492 B5/8 gave 13 fragments and a total estimated molecular weight of 66.7Md compared to strain NZP 5492 B5/1 which had 14 fragments and an estimated total molecular weight of 68.1Md. The data (Plate 49 and Appendix Table IX) show that the plasmids are substantially the same except for the difference of a few bands, shown in Figure 19. The changed bands add up to the same molecular weight in both plasmids.

These changes in the restriction enzyme pattern are indicative of an alteration in the *Eco* RI cutting sites. Alterations which would account for the observed changes are presented in the Discussion (Section 6.1).

PLATE 49: *Eco* RI digests of λ (track 1) *Rhizobium phaseoli* NZP 5492 B5/8 (effective) (track 2) and NZP 5492 B5/1 (ineffective mutant) (track 3)

Electrophoresis was for 6 hours at 60V in a 1% gel.

FIGURE 18: Bonds from plate 49 (*Eco* RI digests of λ, *R. phaseoli* NZP 5492 B5/8 and NZP 5492 B5/1)



49

18

DISCUSSION

1. Plant nodulation tests and selection of spontaneous antibiotic resistant mutants

The work described in this thesis set out to examine the relationship between the plasmid content of various strains of *Rhizobium phaseoli* and their effectiveness in nodulating *Phaseolus vulgaris*.

It was first necessary to verify that the strains to be used were able to nodulate beans and whether the nodules formed were effective in fixing nitrogen. It was possible to decide from plant appearance whether a strain was an effective nodulator or not. Comparison of the weight of tops with that of negative controls (uninoculated plants) gave a numerical value which could be statistically examined by the student-t test. Wet weights of shoots gave a good indication of effectiveness, however the moisture content may have been variable and to eliminate this possible error dry weights of plant tops were used for comparison.

Figure 6 summarises the effectiveness data for the strains used in this investigation. The strains fall into three groups, those which show no significant difference in top dry weight to the uninoculated controls and are therefore ineffective (or non-nodulating), those strains which are fully effective and a group of strains, all from homology group 2 (Crow *et al* 1981) which are not as effective as the fully effective strains.

Uninoculated controls were also used as an indication of contamination. Contamination usually shows first in the uninoculated plants as the contaminant does not face competition from an established strain of *Rhizobium* (Moustafa and Greenwood, 1967). Moustafa and Greenwood considered

that there was little chance of any nodules on the inoculated plants being formed by contaminating rhizobia because of this competition. Rolfe and Gresshoff (1980) in experiments with a mixed ineffective/effective inoculum found that nodules could contain both effective and ineffective bacteria but that each individual plant cell contained either one type or the other but not both. The possibility of mixed infection in a nodule made it necessary to have a means of identifying the *Rhizobium* strain.

Spontaneous resistance to various antibiotics occurs naturally in populations of rhizobia and can be readily selected for (Kondorosi and Johnston 1981). Spontaneous antibiotic resistant mutants are often still fully effective. Schwinghamer and Dudman (1973) tested spectinomycin as an antibiotic marker and found 81% of mutants tested were still fully effective. Pankhurst (1977) used antibiotic resistance as a marker for strain identification. Brockwell *et al* (1977) used streptomycin resistance to identify field test strains. Their results indicated substantial agreement between streptomycin resistance and immunology as techniques for identifying inoculum strains. Schwinghamer (1967) considered streptomycin resistance to be a highly stable characteristic in culture.

However, because spontaneous mutants to one antibiotic could arise in a culture it was decided to use double resistant mutants, that is strains which were simultaneously resistant to two antibiotics. Four antibiotics were tried; Nalidixic acid, streptomycin, spectinomycin and rifampicin. Nalidixic acid at 100µg/cm^3 had little effect on the growth of the organism and was not used.

Streptomycin resistant, Spectinomycin resistant (Sm^r, $Spec^r$); streptomycin resistant, rifampicin resistant (Sm^r, Rif^r) and spectinomycin resistant rifampicin resistant ($Spec^r$, rif^r) mutants of strain NZP 5462 were obtained.

Sm^r rif^r and Sm^r $Spec^r$ mutants of strain 5492 were also obtained. Such double resistant mutants have been used as marked strains in plasmid investigations by other workers, including Brewin *et al* (1980a), Koekman *et al* (1980) and Scott and Ronson (1982).

In our experiments the effectiveness of the double resistant mutants obtained was checked by plant testing and no significant difference was found between the antibiotic sensitive parent strains and the double antibiotic resistant mutants.

2. Isolation of ineffective mutants from antibiotic resistance marked clones.

We were interested in comparing the plasmid composition of an effective strain and an ineffective or non-nodulating mutant obtained from the effective strain. A number of methods have been used for the production of non-nodulating mutants. Chemical mutagenesis has been used to produce non-nodulating mutants, agents used include acridine orange (Higashi 1967) and nitrosoguanadine (Maier and Brill 1976). X-ray and u.v. mutagenesis was used to obtain a number of strains capable of nodulating pea seedlings but incapable of nitrogen fixation (Schwinghamer 1967). MacGregor and Alexander (1971) used u.v. mutagenesis to obtain non-nodulating mutants of *Rhizobium* sp.

These methods all have the disadvantage of damaging the chromosomal DNA as well as the plasmid DNA. We wanted to examine the effect of the plasmid DNA on nodulation and effectiveness. Growth at elevated temperatures was reported to affect the plasmid DNA without affecting other functions. Hamilton and Fall (1971) showed the loss of tumor initiating ability in *Agrobacterium tumefaciens* by incubation at 36°C instead of the normal incubation temperature of 25°C. Zurkowski and Lorkiewicz (1978) obtained

non-nodulating mutants of *R. trifolii* after incubation for 7 days at 35°C. Casse *et al* (1979), after heat treatment of a strain of *R. leguminosarum* obtained a non-nodulating mutant that lacked one of the three large plasmids of the parent strain. Elimination of plasmids from *R. trifolii* by growth at elevated temperature has also been demonstrated (Zurkowski and Lorkiewicz 1979, Scott and Ronson 1982). Curing of plasmids by growth at elevated temperatures has also been demonstrated in *Staphylococci* (May *et al* 1964) and *Proteus* (Terewaki *et al* 1967).

In our experiments growth of *Rhizobium phaseoli* strains NZP 5462 and NZP 5492 at 35°C compared with the normal growth temperature of 25°C failed to yield any non-nodulating mutants. The close similarity between the optical density curves for the two temperatures (Figures 7 and 8) suggests that the elevated temperature was not high enough for the strains used to cause the significant reduction in cell numbers expected (Zurkowski and Lorkiewicz 1978). However a number of small colonies were obtained from these high temperature incubations (Results Section 3). Sanders *et al* (1978) described small colonies which were ineffective in fixing nitrogen, consequently the small colonies obtained from the 35°C temperature treatment were considered worth further investigation. Some of these colonies generated small white ineffective nodules on bean plants as well as red-brown effective nodules. Similar nodules have been described by Buchanan-Wollaston *et al* (1980). If the inoculum from the small colony isolate contained both ineffective and effective rhizobia the presence of ineffective rhizobia in many of the nodules could be masked by that of effective rhizobia. Brewin *et al* (1980) has pointed out the very strong selection for nodulating revertants which exists in a nodulation trial.

Plant testing of the bacteria obtained from the small white nodules yielded both effective red-brown nodules and also a large number of green nodules.

Brewin *et al* (1980) found green nodules which did not reduce acetylene in one clone of a strain of *R. leguminosarum*. Maier and Brill (1976) also described green nodules obtained from nitrosoguanidine induced mutants of *R. japonicum* which did not reduce acetylene. Brill assumed the green appearance was due to the absence of leghemoglobin. Wild type nodules are pink on the inside because of the presence of leghemoglobin (Appleby *et al* 1973).

Hence we have obtained an ineffective mutant of a double antibiotic resistant marked strain of NZP 5492, the ineffectiveness appears to be caused by the absence of leghemoglobin in the nodule. It was then possible to compare the plasmid component of the effective and ineffective mutant of strain NZP 5492.

3. Plasmid extraction methods

All isolation methods for large plasmids include treatments which facilitate the separation of the plasmid DNA from the chromosome-membrane complex. The methods of achieving this separation include physical treatments such as shearing the lysate (Currier and Nester 1976, Nuti *et al* 1977, Zurkowski and Lorkiewicz 1979, Schwinghamer 1980), heat pulse treatment (Hansen and Olsen 1978), chemical treatment such as alkaline lysis (Currier and Nester 1976, Nuti *et al* 1977, Casse *et al* 1979, Kado and Liu 1981) and treatments with various enzymes such as RNAase and pronase (Currier and Nester 1976, Eckhardt 1978).

The success of these separations are dependent on the plasmid being in the covalently closed circular (ccc) or 'supercoiled' form as opposed to the linear chromosomal DNA. Casse *et al* (1979) found that the quantity of plasmid DNA in the supercoiled form was inversely proportional to the size of the plasmid molecule examined. For example plasmid RP4, 36Md molecular weight was found to have 12% in the supercoiled form whereas pRme L5-30 (91 Md) had 8% and pU45 (151 mD) only 5% of total plasmid in the supercoiled form. When large plasmids (100-200Md) are isolated a high proportion of the DNA is obtained in the open circular or linear form, rather than intact ccc DNA. Plasmid DNA in these forms is not observed as a plasmid band on an agarose gel and is not discriminated from linear chromosomal DNA during the extraction protocols. Hence the difficulty of isolating plasmids increases with their size. Casse *et al* (1979) found it necessary to increase the volume of the DNA sample loaded onto the gel in order to detect very large plasmids.

The various protocols described in the literature can be divided into two groups. Firstly 'screening' or small scale methods which are most suitable for examining large numbers of strains or cultures but which do not provide sufficient extracted plasmid to enable further characterisation (such as restriction enzyme analysis).

3.1 Small scale 'screening' methods

These methods were used to demonstrate the presence of plasmids in the *Rhizobium* strains used in this investigation, and to check that the preparative methods used to obtain plasmid DNA for restriction enzyme analysis were giving the same plasmids as could be obtained with a number of more quickly and easily performed methods.

The alkaline lysis method (Birnboim and Doly 1979) (Results section 5.1) was originally developed for *E. coli*. Casse *et al* (1979) considered that the low G.C. content of the *E. coli* chromosomal DNA (50% G.C.) may make it more sensitive to alkaline denaturation than *R. meliloti* or *Agrobacterium* sp. chromosomal DNA (62% G.C.).

In our experiments some *Rhizobium* strains which were known to contain plasmids failed to give any plasmid band (for example NZP 5459, NZP 5462). This was probably due to incomplete lysis and failure to release the large plasmid molecule from the chromosome - membrane complex. The three strains (NZP 5097, NZP 5492 and NZP 5260) successfully examined all had plasmids of the same relative mobility. The method cannot produce sufficient DNA for a more detailed examination and was not further investigated.

The method of Anderson *et al* (1981) (Results Section 5.2) has been used successfully to extract *Rhizobium* sp. plasmids (D.B. Scott, personal communication). Our experiments showed that lysis at room temperature produced too much linear DNA which masked the plasmid bands. Lysis on ice which was less complete was therefore preferred. Plasmids of similar molecular size were demonstrated in two DNA homology group 2 strains, NZP 5462 and NZP 5456. The relative mobility of these plasmids indicated that they were of similar size to the DNA homology group 1 plasmids from strains NZP 5492, NZP 5097 and NZP 5260. Sufficient plasmid DNA to enable further characterisation was not obtainable with this method.

Because the Eckhardt (1978) method is performed in the well of the gel the released plasmid is less harshly treated before being electrophoresed. For example there is no stirring or shearing which tends to break up large plasmids. Procedures which involve the least number of destructive operations (stirring, pipetting, precipitation and resuspension) allow recovery of the largest plasmids (Denarie *et al*

1981). The Eckhardt method therefore had the potential to isolate much larger plasmids than methods such as Currier and Nester. It was therefore not unexpected that more plasmids were observed when this method was applied to strain NZP 5462. The Eckhardt method proved difficult to handle on horizontal gel apparatus and since it was unable to produce sufficient plasmid DNA for further characterisation it was not continued with.

The method of Casse *et al* (1979) had the possibility of scaling up to produce sufficient plasmid DNA to permit characterisation by restriction enzymes. However, although the small scale method was successful, the large scale methods were abandoned because we were unable to obtain good yields of plasmid DNA.

3.2 Currier and Nester Method

The method of Currier and Nester (Results Section 5.3) was successfully used to obtain plasmid DNA from all *Rhizobium phaseoli* strains to which it was applied. This method used relatively large volumes of cell culture (litre quantities) and corresponding volumes of reagents such as phenol and chloroform. It was therefore relatively time consuming and expensive to perform. The method also contains a shearing step which may result in damage to plasmid molecules (Schwinghamer 1980, Denarie *et al* 1981). There is a narrow range of pH between about 12.0 to 12.5 in which denaturation of linear DNA but not ccc DNA occurs. This property has been used for purifying ccc DNA (Currier and Nester 1976, Kado and Liu 1979, Birnboim and Doly 1979, Casse *et al* 1979, Hirsch *et al* 1980). For reproducible isolation of large plasmids our results showed that accurate control of the pH of the lysate during the alkali denaturation and neutralization step was essential. If the pH of the lysate was not high enough for a sufficiently long time the chromosomal DNA was not denatured and overloaded the preparation.

If the pH was too high or was held at the right level for too long the supercoiled plasmid became damaged and plasmid yield was reduced. The main difficulties encountered with this method were control of the shearing step and the accurate adjustment of the reaction pH in the slightly viscous lysate during the alkali denaturation step. Despite the difficulties the number of plasmid bands observed with this method was identical to that of other methods tried (with the exception of the Eckhardt method, previously discussed). Since this method resulted in relatively large quantities of crude plasmid DNA it (or the Koekman variation (Method 5.92) were the methods of choice for the preparative isolation of plasmid DNA for further purification. The Currier and Nester method has been used with slight modifications by Costantino *et al* (1980) and Garfinkel and Nester (1980) for the preparative isolation of Ti plasmid DNA from *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* . Costantino's modifications related to the concentration of cells in the resuspension and the use of pronase E instead of pronase B. Our experience was that either pronase E or B was satisfactory for the lysis of *R. phaseoli* strains. Garfinkel and Nester used 0.3M sodium acetate in place of magnesium chloride and sodium phosphate in the DNA precipitation step. Sodium acetate causes the mass of DNA in solution to aggregate and form an insoluble network (Birnboim and Doly 1979). However both sodium acetate and ethanol precipitation promote the co-precipitation of protein contaminants with the DNA which makes the pellet difficult to resuspend.

3.3 Koekman Modification

The difficulty of co-precipitation of protein contaminants was overcome by the use of Koekman's modification of the Currier and Nester method, (Results Section 5.4) which uses polyethylene glycol (PEG) to precipitate the DNA. The precipitation is dependent on the PEG concentration and

independent of the molecular weight of the plasmid. PEG precipitation of DNA is fairly rapid being 90% complete in 2 hours at 4°C, and is very gentle, causing no change in the distribution of covalently closed circular, open circular or linear DNA (Humphreys *et al* 1975). PEG precipitation has been used for the recovery of large plasmids of 250-300 Md molecular weight from *Pseudomonas aeruginosa* (Hansen and Olsen 1978), *Agrobacterium tumefaciens* and *Rhizobium leguminosarum* (Hirsch *et al* 1980) and *Rhizobium trifolii* (Beynon *et al* 1980). Because proteins are not co-precipitated with PEG the deproteinization step using a phenol-chloroform extraction can be avoided. This reduces mechanical handling of the plasmid containing solutions and results in a quicker and more conveniently performed method. The Koekman modification of the Currier and Nester method was the preferred method for preparing crude plasmid extracts for purification. This method has been used by Hooykaas *et al* (1980) for the isolation of Ti plasmid DNA from *Agrobacterium tumefaciens*.

3.4 Kado and Liu Method

This method has been used on some *Rhizobium meliloti* strains by Kado and Liu and is potentially capable of producing chromosomal free plasmid DNA in sufficient quantities for restriction enzyme digestion. It is also relatively quick and simple to perform, consequently, some time was spent modifying the basic steps in the method to improve the yield of plasmid DNA from the strains of *Rhizobium* under investigation. It was found that the efficiency with which plasmid DNA was recovered was dependent on the bacterial strain and on the number of cells used (Results Section 5.51). Schwinghamer (1980) has stated 'Susceptibility to lysis may vary considerably between strains of a species and between the phases of growth of a culture' Kado and Liu (1979) found that good lysis seemed to depend on the stage of cell growth and on the temperature and length of incubation. Our experiments verified these findings and showed

that the temperature treatment was important in reducing the amount of chromosomal DNA in the preparation (Results Section 5.52). The 'smeared' region found in many preparations was probably due to degredation of the plasmid DNA since further degredation of the linear chromosomal DNA would have resulted in a band which migrated more rapidly rather than less rapidly through the agarose gel (Meyers *et al* 1976). We found a correlation between the loss of the higher molecular weight plasmid in some preparations, and the increase in the smeared region. Kado claimed that heat treatment of *Agrobacterium* strains at 95°C for 5 mins was effective in complete chromosome elimination but we found that this treatment removed all DNA including the large plasmids.

Some difficulty was experienced in measuring the pH of the lysing solution in the pH 12.5 region. A combination of pH meter and indicator sticks (Method 5.6) were used however lysing solutions were most reproducibly prepared by using measured volumes of 2M NaOH in carbon dioxide free water to achieve the required pH. The pH of the lysing solution was considered by Kado and others (D.B. Scott, personal communication) to be important, however we found little effect on raising the pH to 13.0 and above (Results Section 5.54). This may be because denaturation of ccc molecules is reversible because of the intertwisting of both strands (Denarie *et al* 1981) hence plasmid yield is not as affected by higher pH's as may have been expected.

Salt treatment was thought to aid precipitation of the chromosome - membrane complex and its removal in the phenol extraction, however complete chromosomal DNA elimination was not achieved with any of the modifications attempted, namely heat treatment, salt treatment or high pH, hence the Kado method was discarded in favour of using a large scale method such as Currier and Nester or the Koekman modification followed by a purification step.

4. Purification of plasmid DNA by gradient centrifugation

Plasmid DNA has been separated from chromosomal DNA by hydroxyapatite columns (Colman *et al* 1978),use of specific DNA absorbant columns (Buenemann and Mueller 1978) elution from sea plaque agarose gels (Weislander 1979, Langridge *et al* 1980), sucrose gradient ultracentrifugation (Ledeboer *et al* 1976) and cesium chloride density gradient centrifugation (Currier and Nester 1976, Zurkowski and Lorkiewicz 1979, Ledeboer *et al* 1976, Rosenberg *et al* 1981). We chose gradient centrifugation because it was applicable to handling the volumes of plasmid generated from the extraction methods chosen and because the resources to perform the methods were available.

4.1 Sucrose gradients

Ledeboer *et al* (1976) used 5-20% neutral sucrose gradients to purify Ti plasmid from *Agrobacterium tumefaciens*. Ledeboer used ^{3}H-Thymidine labelled cells which enabled the separation of relatively dilute DNA solutions on the sucrose gradient. We found that alteration of the gradient density from 5-20% to 10-30% and/or increasing the period of centrifugation increased the separation but this was not complete with DNA solutions of 2mg/cm^{3} although more dilute (0.2mg/cm^{3}) solutions could be separated (Results Section 6.1). We were unable to separate plasmid from chromosomal DNA at the concentration required to enable further use of the separated plasmid DNA.

4.2 Cesium chloride gradients

Cesium chloride-ethidium bromide density gradients (Bauer and Vinograd 1968) have been used by many workers to separate viral and plasmid DNA and the technique has been successfully applied to large *Agrobacterium* and *Rhizobium* plasmids

(Currier and Nester 1976, Zurkowski and Lorkiewicz 1979, Ledeboer *et al* 1976, Rosenberg *et al* 1981).

Fixed angle rotors were used because they were reported to give improved separation of the plasmid and chromosomal bands over swinging bucket rotors (Freifelder 1971). Cesium chloride gradient centrifugation is more time consuming than sucrose gradient centrifugation (Section 4.1) however the use of vertical rotors is reported to reduce the time taken to as little as two hours (Wells and Brunk 1979). Excellent correlation was found between the theoretical gradient (Methods Section 6.25) and the actual gradient formed (Results Section 6.2 and Figure 12). The plasmid and chromosomal bands were sufficiently separated in the gradient to allow purification (plate 44). Plasmid DNA obtained in this way was used for restriction enzyme analysis (Results Section 8). Some conversion of the ccc form to oc and linear forms normally took place during the recovery of the plasmid from the gradient. This was minimised by careful avoidance of steps which could cause mechanical damage and by preventing ethidium bromide containing plasmid solutions from becoming exposed to uv irradiation. The gradient conditions which we used did not separate individual plasmids from each other. It may be possible to separate plasmids of different molecular size in a sucrose gradient after first separating them from chromosomal DNA in a cesium chloride gradient.

5. Estimation of the molecular weight of whole plasmids

The molecular weight of whole plasmids was estimated by comparing their relative mobility in an agarose gel to that of reference plasmids of known molecular weight (Results Section 7). Meyers *et al* (1976) found that estimation of plasmid molecular weights from the extent of migration of the covalently closed circular DNA band in agarose gels compared favourably with results obtained by electron microscopy of the plasmid DNA purified by cesium chloride-ethidium

bromide density gradient centrifugation. Meyers found a linear relationship between the logarithm of plasmid molecular weight and the logarithm of the mobility of the plasmid for plasmids ranging from 1.87Md to 93.2Md. Casse *et al* (1979) examined plasmids between 91Md and 140Md and found that the same correlation between $\log_{10}$ molecular weight and $\log_{10}$ mobility applied. Above 140Md molecular weights were calculated by linear extrapolation of the curve obtained in the 90Md to 140Md region and this resulted in underestimation of molecular size. Hansen and Olsen (1978) reported that agarose gel electrophoresis estimates of very large plasmids (312Md and 280Md) were underestimated. One of the problems we found in constructing a standard curve was obtaining suitable plasmids of verified molecular weight in the region 60Md to 150Md, nevertheless a plot of $\log_{10}$ molecular weight against $\log_{10}$ relative plasmid mobility for the reference plasmids we had gave a straight line (Figure 14), from which we were able to estimate the molecular weights of the unknown plasmids (Table XXII). The *Rhizobium phaseoli* strains contained plasmids ranging from 66Md to 316Md.

5.1 *Rhizobium phaseoli* NZP 5479 and 5547

Rhizobium phaseoli NZP 5547 is a completely non-nodulating mutant of *Rhizobium phaseoli* NZP 5479 (Results Section 4). We obtained two plasmids from each of these strains, estimated molecular weights 186Md and 288Md. There was no detectable difference in the size of the plasmids in the non-nodulating mutant compared to the effective parent strain.

Casse *et al* 1979 obtained a *R. leguminosarum* non-nodulating mutant. This mutant lacked one of the three large plasmids found in the parent strain. Loss of the nod$^+$ phenotype has also been correlated with the loss of plasmids by Zurkowski and Lorkiewicz (1978), Prakash *et al* (1978), Hooykaas *et al* (1981) and Scott and Ronson (1982). Loss

of nodulation ability has also been shown to be due to a deletion within a plasmid (Beynon *et al* 1980; Denarie *et al* 1981, Hirsch *et al* 1980, Kondorosi *et al* 1981).

If the plasmids of this strain are involved in nodulation the mutation must involve a change in a plasmid not observed in our preparations (probably of greater than 350Md molecular weight) or a rearrangement of the DNA within the existing plasmid structure so that the total plasmid size is not altered, or a very small deletion which does not cause a detectable size change.

6. Restriction endonuclease digests

We wished to compare purified plasmid DNA from *R. phaseoli* strains within DNA homology group 1 to see if the plasmids were the same or similar and then to compare these plasmids with those obtained from strains in homology group 2.

Restriction endonucleases have been used by many workers to compare plasmid DNA in *Agrobacterium* strains (Koekman *et al* 1980, Garfinkel and Nester 1980, Hooykaas *et al* 1980, Costantino *et al* 1980). Restriction endonucleases used include *Eco* RI, *Bam* HI, *Hin*dIII, *Sal* I, *Pst* I, *Hpa* I, *Kpn* I and *Sma* I.

In our experiments we used *Eco* RI, *Bam* HI and *Hin*d III. The molecular weights of the fragments obtained were estimated as for the whole plasmids (Section 5). Accurate determination of the plasmid molecular weight by this method depends on detection of all the plasmid fragments. If two fragments are of similar molecular size they will migrate at the same rate on the agarose gel and will only appear as one band. This will have a negligible effect for fragments of small size but large molecular weight fragments masked in this way may have a considerable effect on the estimated molecular weight. Reproducibility between digests requires that the plasmid be fully digested in each digest. If partial digest-

ion occurs high molecular weight bands may appear which would disappear if the digest went to completion. However, in a partial digest some of the plasmid will be completely digested, thus a higher molecular weight will be estimated for a partial digest.

All of the *R. phaseoli* strains we examined contained two plasmids. If we did not obtain complete separation of the two plasmids the fragments obtained from digests could vary depending on the amount of contamination with the second plasmid.

The total molecular weight of the plasmid from *R. phaseoli* NZP 5492 shows some variance depending on the restriction enzyme used (Table XXIII). The values range from 51.6Md to 77.3Md with a mean of 64.6Md compared to 66Md for the whole plasmid. The 77.3Md estimate is from 19 fragments and the lower estimate from 13 fragments. An explanation for the differences may be found in the factors discussed above. Digest number 3 (Table XXIII) gives higher molecular weight estimates for all of the plasmids. It may be that the plasmids were incompletely digested in this digest.

The DNA homology group 1 strains NZP 5492 and NZP 5097 differ in both the total number of fragments obtained from a digest and in the molecular weights of these fragments. It is not possible from this information to tell whether the plasmids have significant regions of DNA homology with each other and thus whether they may code for similar functions. To show this it would be necessary to undertake blotting and hybridization studies similar to those used on Ti plasmids by Tomashow *et al* (1981), White and Nester (1980) and Schweitzer *et al* (1980).

Similarly the fragment patterns of the DNA homology group 1 strains differ from that of the DNA homology group 2 strain.

The number of fragments (22) obtained for the plasmid from NZP 5462 (DNA homology group 2) and NZP 5097 (DNA homology group 1) are similar. To determine whether the DNA sequences are similar it is necessary to hybridize a plasmid from one DNA homology group to a blot containing a digest of plasmids from the other DNA homology group. This would demonstrate if regions of homology exist between the plasmids of the two homology groups.

6.1 Comparison of *R. phaseoli* NZP 5492 B5/8 (effective) and the ineffective mutant NZP 5492 B5/ 1

These two strains each contained two plasmids of the same molecular size. The lower molecular weight plasmid was digested (plate 49) in each case. The total molecular weights of the digest fragments were very similar (66.7Md and 68.1Md). Strain NZP 5492 B5/8 had 13 fragments strain NZP 5492 B5/1 had 14 fragments. The fragment pattern was very similar but 3 bands in the NZP 5492 B5/8 digest totaling 21.5Md and 4 bands in the NZP 5492 B5/1 digest totaling 21.7Md were different (Figure 19). The dissimilar fragments in the two digests added up to the same molecular weight in each case. Changes in the fragment pattern could be explained by the addition of one *Eco* RI site in the ineffective mutant and a shift in another. Figure 20 gives two possible rearrangements of the DNA which would account for the observed changes in the fragment pattern. It is not possible from this information to determine if the change in band pattern is the cause of the ineffectiveness of the mutant.

7. Conclusion

In the introduction it was postulated that *Rhizobium* strains from the different homology groups may be able to nodulate beans because they contain a common plasmid. We have shown

FIGURE 19: *Eco* RI digests of *Rhizobium phaseoli* NZP 5492 B5/8 (left) and NZP 5492 B5/1 (right) showing the fragments which differ between the effective and ineffective strain

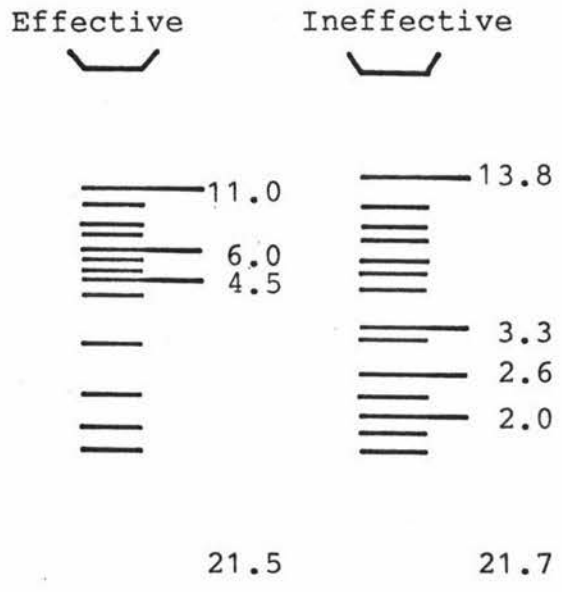
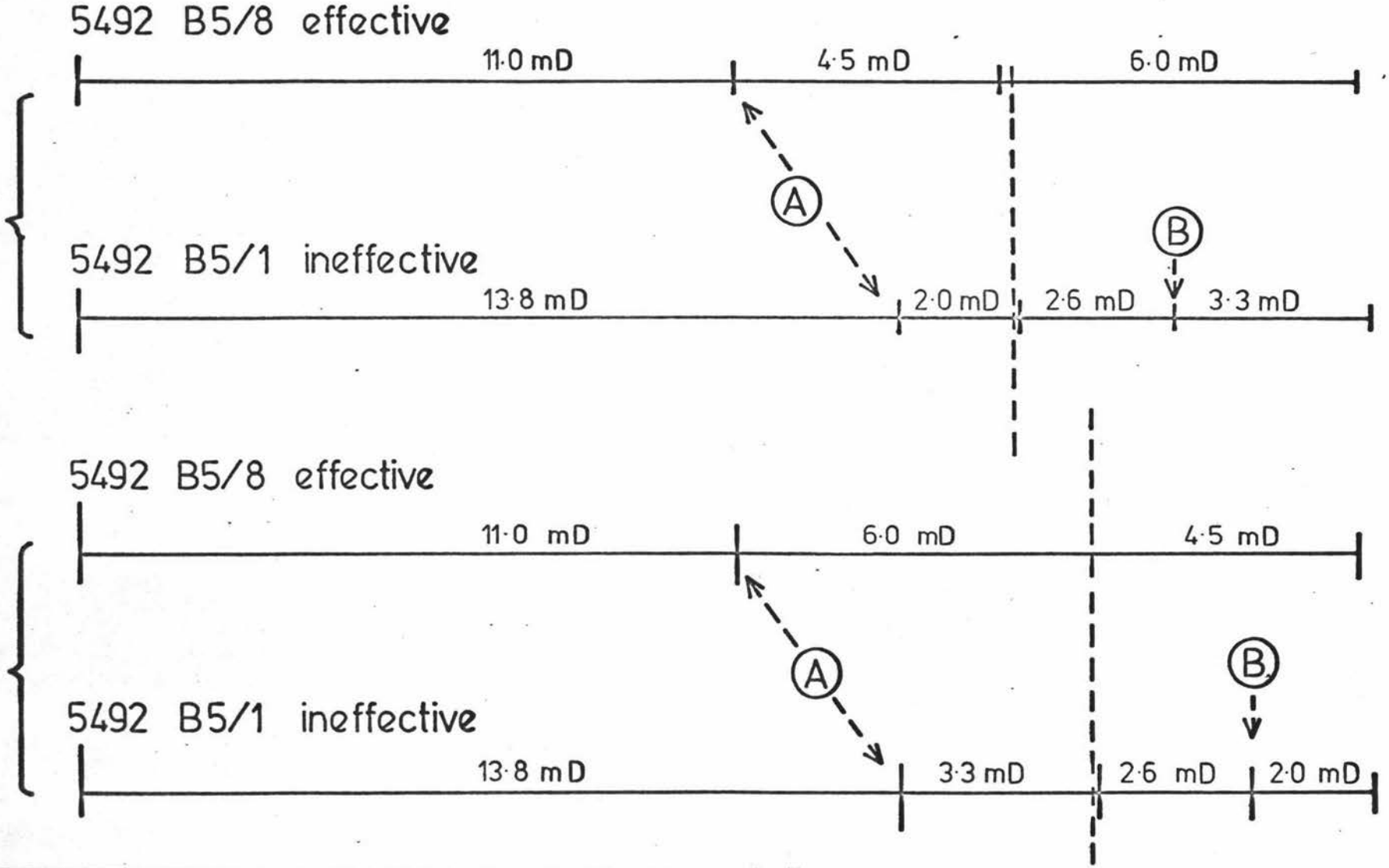


FIGURE 20: Diagram showing possible changes in the plasmid DNA giving rise to the observed changes in the fragment pattern.

(A) shift in an *Eco* RI site
(b) additional *Eco* RI site


5492 B5/8 effective
11·0 mD
4·5 mD
6·0 mD
A
B
5492 B5/1 ineffective
13·8 mD
2·0 mD
2·6 mD
3·3 mD
5492 B5/8 effective
11·0 mD
6·0 mD
4·5 mD
A
B
5492 B5/1 ineffective
13·8 mD
3·3 mD
2·6 mD
2·0 mD

that the plasmids within one homology group are not the same and that they are not similar to a plasmid from the second homology group. Thus the simple hypothesis that the plasmids are the same is not true, although the plasmids may contain common sequences which enable them to nodulate beans.

Our work leads us to conclude that small genetic rearrangements in the plasmid DNA may have a marked effect on the phenotype of the bacteria. Genome rearrangements have recently been demonstrated in the Rhizobiaceae (W. Heumann, unpublished). It is possible that the differences in plasmids have arisen by rearrangement with the chromosomal DNA. This may explain why genes for nodulation and nitrogen fixation are sometimes plasmid borne and sometimes found on the chromosome in *Rhizobium* . Genome rearrangements between plasmid and chromosomal DNA may also explain the wide plant specificity of some *Rhizobium* strains.

APPENDIX

The molecular weights and mobility of fragments from restriction enzyme digests of λ and *Rhizobium phaseoli* plasmids.

TABLE A1: THE MOLECULAR WEIGHTS AND MOBILITY OF FRAGMENTS OBTAINED BY DIGESTING λ WITH *Eco* RI, *Hin*dIII AND *Bam* HI (plate 46).

	molecular weight of fragment	$\log_{10}$ molecular weight	relative mobility of fragment	$\log_{10}$ relative mobility
Eco RI	13.7	1.14	1.74	0.24
	4.5	0.65	2.50	0.40
	3.5	0.54	2.84	0.45
	3.0	0.48	3.07	0.49
	2.3	0.36	3.66	0.56
*Hin*dIII	14.4	1.16	1.75	0.24
	6.2	0.80	2.26	0.35
	4.2	0.62	2.67	0.43
	2.8	0.45	3.24	0.51
	1.6	0.20	4.54	0.66
	1.4	0.15	4.87	0.69
Bam HI	11.5	1.06	1.90	0.28
	4.8	0.68	2.11	0.32
	4.5	0.65	2.56	0.41
	4.3	0.63	2.77	0.44
	3.7	0.57	2.96	0.47

FIGURE A1: Standard curve relating $\log_{10}$ relative mobility to $\log_{10}$ molecular weight for λ fragments of known molecular weight obtained by digestion with *Eco* RI, *Hin*dIII and *Bam*HI (plate 46)

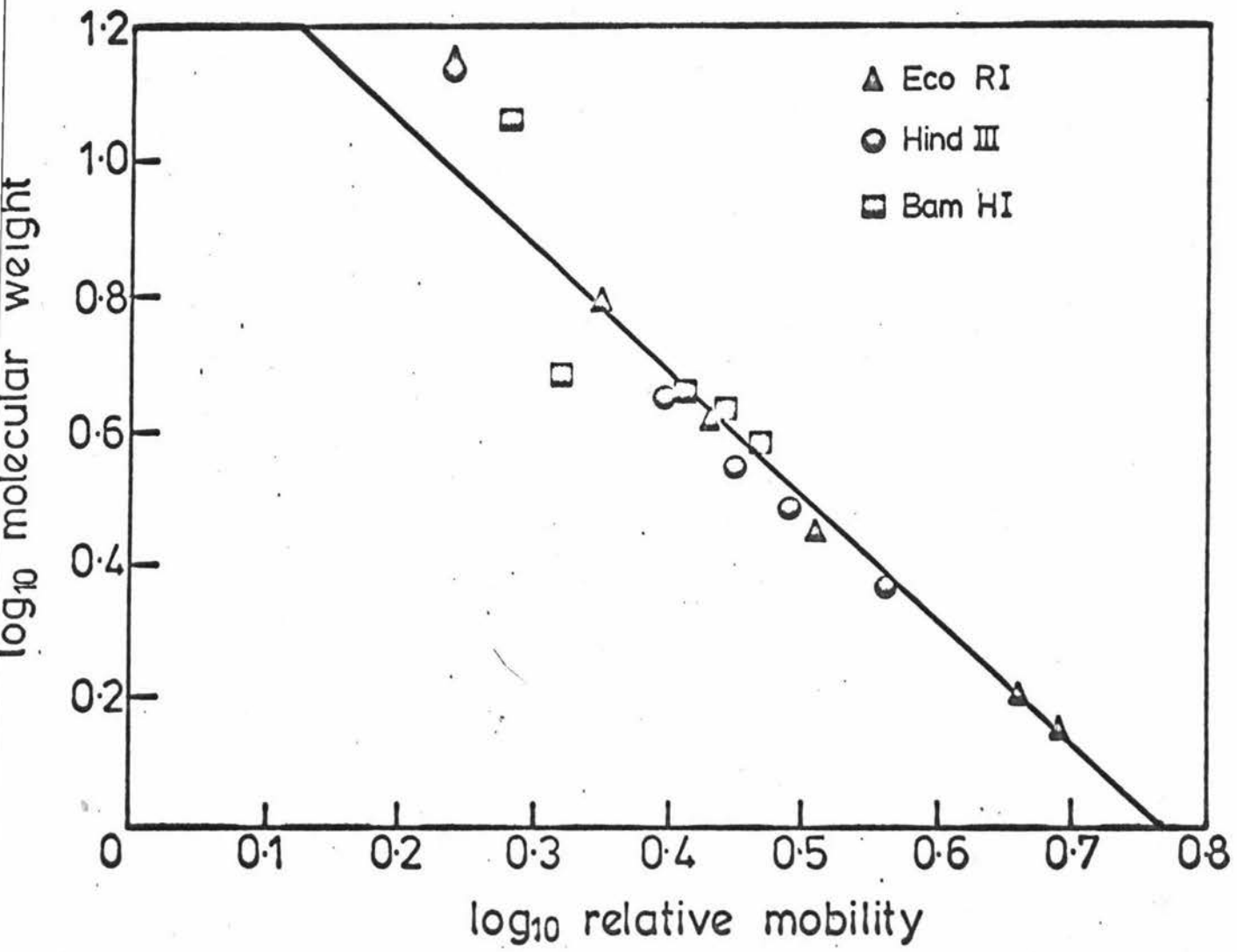


TABLE AII: The relative mobilities and estimated molecular weights of fragments obtained by digesting the plasmid from *Rhizobium phaseoli* NZP 5492 with *Eco* RI, *Hin*dIII and *Bam* HI (plate 46)

relative mobility of fragment	$\log_{10}$ relative mobility	$\log_{10}$ estimated molecular weight	estimated fragment molecular weight (Md)
Eco RI	15 fragments:	estimated molecular weight	56.4Md
1.90	0.28	0.90	7.9
2.12	0.33	0.81	6.5
2.20	0.34	0.79	6.2
2.29	0.36	0.76	5.8
2.44	0.39	0.70	5.0
2.55	0.41	0.66	4.6
2.66	0.42	0.64	4.4
3.22	0.51	0.48	3.0
3.50	0.54	0.42	2.6
3.61	0.56	0.38	2.4
3.94	0.60	0.30	2.0
4.13	0.62	0.27	1.9
4.26	0.63	0.25	1.8
4.99	0.70	0.12	1.3
6.0	0.78	0.00	1.0
*Hin*dIII	19 fragments:	estimated molecular weight	55.4Md
2.27	0.36	0.75	5.6
2.38	0.38	0.72	5.3
2.56	0.41	0.66	4.6
2.66	0.42	0.64	4.4
2.73	0.44	0.61	4.1
2.89	0.46	0.57	3.7
3.18	0.50	0.49	3.1
3.28	0.52	0.46	2.9
3.41	0.53	0.44	2.8
3.51	0.55	0.40	2.5
3.82	0.58	0.34	2.2
3.92	0.59	0.32	2.1

Cont'd...

relative mobility of fragment	$\log_{10}$ relative mobility	$\log_{10}$ estimated molecular weight	estimated fragment molecular weight (Md)
4.80	0.68	0.16	1.4
5.08	0.71	0.10	1.3
5.32	0.73	0.08	1.2
5.49	0.74	0.04	1.1
5.66	0.75	0.03	1.1
5.88	0.77	0.00	1.0
6.06	0.78	0.00	1.0
Bam HI	19 fragments - estimated molecular weight 77.3Md		
1.63	0.21	1.02	10.5
1.77	0.25	0.96	9.1
2.02	0.31	0.85	7.1
2.20	0.34	0.79	6.2
2.28	0.36	0.76	5.8
2.43	0.39	0.70	5.0
2.55	0.41	0.66	4.6
2.63	0.42	0.64	4.4
2.72	0.43	0.62	4.2
2.83	0.45	0.59	3.9
3.15	0.50	0.49	3.1
3.42	0.53	0.44	2.7
3.69	0.57	0.36	2.3
4.00	0.60	0.3	2.0
4.54	0.66	0.20	1.6
4.80	0.68	0.16	1.4
5.04	0.70	0.12	1.3
5.67	0.75	0.02	1.1
6.23	0.79	0.00	1.0

TABLE AIII: The relative mobilities and estimated molecular weights of fragments obtained by digesting the plasmid from *Rhizobium* sp. NZP 5462 with *Eco* RI (plate 46)

relative mobility of fragment	$\log_{10}$ relative mobility	$\log_{10}$ estimated molecular weight	estimated fragment molecular weight (Md)
2.22	0.35	0.77	5.9
2.36	0.37	0.74	5.5
2.48	0.39	0.70	5.0
2.72	0.43	0.63	4.3
2.85	0.45	0.59	3.9
2.94	0.47	0.55	3.5
3.02	0.48	0.53	3.4
3.13	0.50	0.49	3.1
3.30	0.52	0.46	2.9
3.46	0.54	0.42	2.6
3.77	0.58	0.36	2.3
3.94	0.60	0.31	2.0
4.21	0.62	0.27	1.9
4.31	0.63	0.25	1.8
4.44	0.65	0.21	1.6
4.58	0.66	0.20	1.6
4.77	0.68	0.15	1.4
5.04	0.70	0.12	1.3
5.16	0.71	0.10	1.3
5.25	0.72	0.08	1.2
5.92	0.77	0.00	1.0
6.07	0.78	0.00	1.0
6.35	0.80	0.00	1.0

23 fragments; estimated molecular weight 61.5Md

TABLE AIV: The molecular weights and mobility of fragments obtained by digesting λ with *Eco* RI (plate 47)

molecular weight of fragment (Md)	$\log_{10}$ molecular weight	relative mobility of fragment	$\log_{10}$ relative mobility
13.7	1.14	2.58	0.41
4.5	0.65	3.82	0.58
3.5	0.54	4.25	0.63
3.0	0.48	4.55	0.66
2.3	0.36	5.09	0.71

FIGURE A2: Standard curve relating $\log_{10}$ relative mobility to $\log_{10}$ molecular weight for λ fragments of known molecular weight obtained by digestion with *Eco* RI (plate 47)

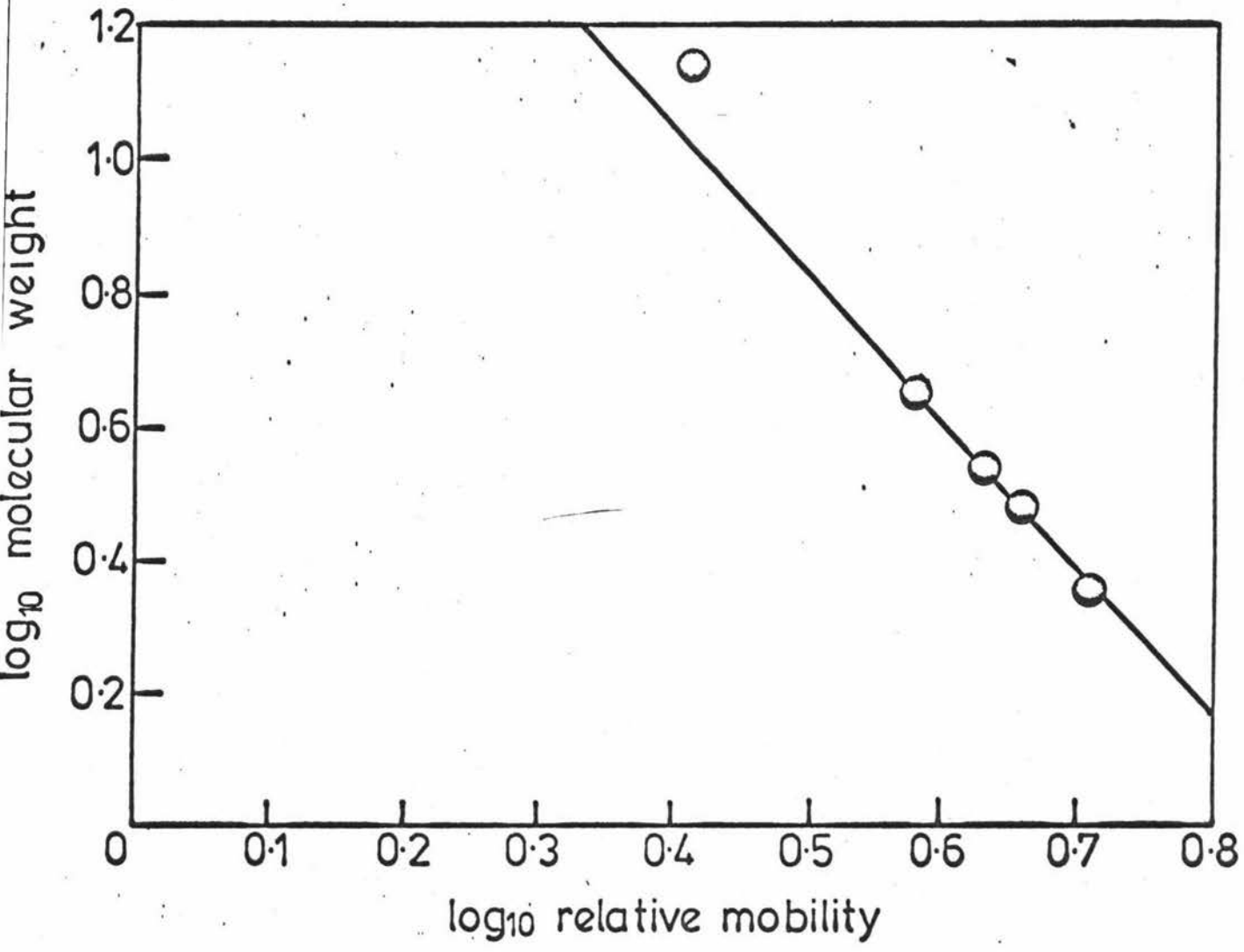


TABLE AV: The relative mobilities and estimated molecular weights of fragments obtained by digesting plasmids from *Rhizobium phaseoli* NZP 5492 and NZP 5097 and *Rhizobium* sp. NZP 5462 with *Eco* RI (plate 47)

relative mobility of fragment	$\log_{10}$ relative mobility	$\log_{10}$ estimated molecular weight	estimated fragment molecular weight (Md)
Rhizobium phaseoli NZP 5492; 13 fragments, estimated molecular weight 51.6Md			
2.80	0.45	0.93	8.5
3.20	0.51	0.80	6.3
3.29	0.52	0.78	6.0
3.43	0.54	0.74	5.5
3.58	0.55	0.71	5.1
3.77	0.58	0.65	4.5
4.01	0.60	0.60	4.0
4.55	0.66	0.47	3.0
5.04	0.70	0.38	2.4
5.47	0.74	0.30	2.0
5.74	0.76	0.25	1.8
6.48	0.81	0.14	1.4
7.33	0.87	0.12	1.1
Rhizobium phaseoli NZP 5097; 21 fragments; estimated molecular weight 62.8Md			
3.02	0.48	0.86	7.2
3.18	0.50	0.82	6.6
3.38	0.53	0.76	5.8
3.57	0.55	0.71	5.1
3.69	0.57	0.67	4.7
3.85	0.59	0.62	4.2
4.02	0.60	0.60	4.0
4.26	0.63	0.54	3.5
4.43	0.65	0.49	3.1
4.84	0.68	0.43	2.7
5.14	0.71	0.36	2.3

Cont'd...

relative mobility of fragment	$\log_{10}$ relative mobility	$\log_{10}$ estimated molecular weight	estimated fragment molecular weight (Md)
5.43	0.73	0.32	2.1
5.75	0.76	0.25	1.8
6.00	0.78	0.21	1.6
6.21	0.80	0.16	1.4
6.75	0.83	0.10	1.3
6.92	0.84	0.08	1.2
7.18	0.86	0.04	1.1
7.44	0.87	0.02	1.1
7.78	0.89	0.00	1.0
8.03	0.90	0.00	1.0
Rhizobium sp NZP 5462; 23 fragments, estimated molecular weight 65.1Md			
3.24	0.51	0.80	6.3
3.40	0.53	0.76	5.8
3.56	0.55	0.71	5.1
3.68	0.57	0.67	4.7
3.92	0.59	0.62	4.2
4.04	0.61	0.58	3.8
4.18	0.62	0.56	3.6
4.29	0.63	0.54	3.5
4.44	0.65	0.49	3.1
4.55	0.66	0.47	3.0
4.70	0.67	0.45	2.8
4.90	0.69	0.41	2.6
5.19	0.72	0.34	2.2
5.41	0.73	0.32	2.1
5.69	0.76	0.25	1.8
5.88	0.78	0.21	1.6
6.16	0.79	0.18	1.5

Cont'd..

relative mobility of fragment	$\log_{10}$ relative mobility	$\log_{10}$ estimated molecular weight	estimated fragment molecular weight (Md)
6.51	0.81	0.14	1.4
7.12	0.85	0.06	1.2
7.35	0.87	0.02	1.1
7.73	0.89	0.00	1.0
7.98	0.90	0.00	1.0

TABLE AVI: The molecular weights and mobility of fragments obtained by digesting λ with *Eco* RI (plate 48)

molecular weight of fragment (Md)	$\log_{10}$ molecular weight	relative mobility of fragment	$\log_{10}$ relative mobility
13.7	1.14	1.35	0.10
4.5	0.65	2.19	0.34
3.5	0.55	2.53	0.40
3.0	0.48	2.85	0.46
2.3	0.36	3.45	0.54

FIGURE A3: Standard curve relating $\log_{10}$ relative mobility to $\log_{10}$ molecular weight for λ fragments of known molecular weight obtained by digestion with *Eco* RI (plate 48)

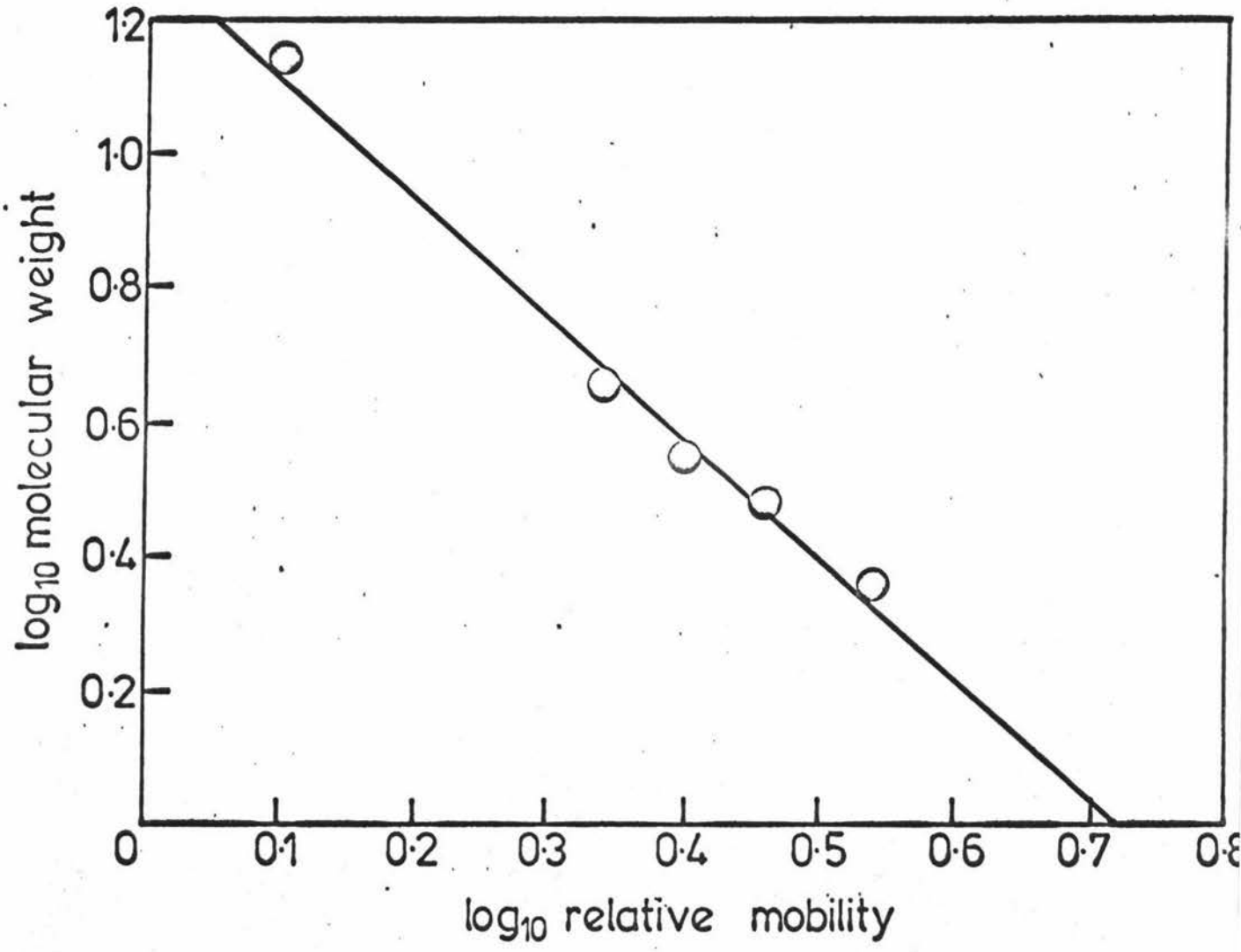


TABLE AVII: The relative mobilities and estimated molecular weights of fragments obtained by digesting plasmids from *Rhizobium phaseoli* NZP 5492 and NZP 5097 and *Rhizobium* sp. NZP 5462 with *Eco* RI (plate 48)

relative mobility of fragment	$\log_{10}$ relative mobility	$\log_{10}$ estimated molecular weight	estimated fragment molecular weight (Md)
Rhizobium phaseoli NZP 5492; 16 fragments; estimated molecular weight 76.9Md			
1.30	0.11	1.10	12.6
1.46	0.16	1.00	10.0
1.68	0.23	0.88	7.6
1.77	0.25	0.84	6.9
1.86	0.27	0.80	6.3
1.96	0.29	0.77	5.9
2.10	0.32	0.72	5.2
2.19	0.34	0.65	4.5
2.34	0.37	0.62	4.2
2.83	0.45	0.48	3.0
3.26	0.51	0.38	2.4
3.39	0.53	0.34	2.2
3.73	0.57	0.26	1.8
4.00	0.60	0.22	1.7
4.14	0.62	0.18	1.5
4.84	0.68	0.06	1.1
Rhizobium phaseoli NZP 5097; 23 fragments, estimated molecular weight 94.5Md			
1.10	0.04	1.22	16.6
1.38	0.14	1.02	10.5
1.67	0.22	0.90	8.0
1.77	0.25	0.84	6.9
1.87	0.27	0.80	6.3
1.98	0.29	0.77	5.9
2.09	0.32	0.72	5.2

Cont'd..

relative mobility of fragment	$\log_{10}$ relative mobility	$\log_{10}$ estimated molecular weight	estimated fragment molecular weight (Md)
2.20	0.34	0.68	4.8
2.34	0.37	0.64	4.4
2.55	0.41	0.56	3.6
2.83	0.45	0.48	3.0
3.17	0.50	0.40	2.5
3.50	0.54	0.32	2.1
3.60	0.56	0.29	2.0
3.76	0.58	0.25	1.8
3.89	0.59	0.24	1.7
4.03	0.60	0.22	1.7
4.16	0.62	0.18	1.5
4.34	0.64	0.14	1.4
4.48	0.65	0.12	1.3
4.64	0.67	0.09	1.2
4.99	0.70	0.02	1.1
5.14	0.71	0.01	1.0
Rhizobium sp. NZP 5462; 21 fragments, estimated molecular weight 94.7Md			
1.12	0.05	1.2	15.8
1.41	0.15	1.02	10.5
1.51	0.18	0.96	9.1
1.68	0.23	0.87	7.4
1.80	0.26	0.82	6.6
1.87	0.27	0.80	6.3
2.01	0.30	0.75	5.6
2.34	0.37	0.62	4.2
2.55	0.41	0.56	3.6
2.66	0.42	0.54	3.5
2.88	0.46	0.48	3.0
3.03	0.48	0.43	2.7
3.17	0.50	0.39	2.5
3.25	0.51	0.38	2.4

Cont'd..

relative mobility of fragment	$\log_{10}$ relative mobility	$\log_{10}$ estimated molecular weight	estimated fragment molecular weight (Md)
3.46	0.54	0.32	2.1
3.58	0.55	0.30	2.0
3.79	0.58	0.25	1.8
4.02	0.60	0.22	1.7
4.32	0.64	0.15	1.4
4.47	0.65	0.12	1.3
4.68	0.67	0.09	1.2

TABLE AVIII: The molecular weights and mobility of fragments obtained by digesting λ with *Eco*RI (plate 49)

molecular weight of fragment (Md)	$\log_{10}$ molecular weight	relative mobility of fragment	$\log_{10}$ relative mobility
13.7	1.14	1.58	0.20
4.5	0.65	2.80	0.45
3.5	0.55	3.23	0.51
3.0	0.48	3.60	0.56
2.3	0.36	4.33	0.64

FIGURE A4: Standard curve relating $\log_{10}$ relative mobility to $\log_{10}$ molecular weight for λ fragments of known molecular weight obtained by digestion with *Eco* RI (plate 49)

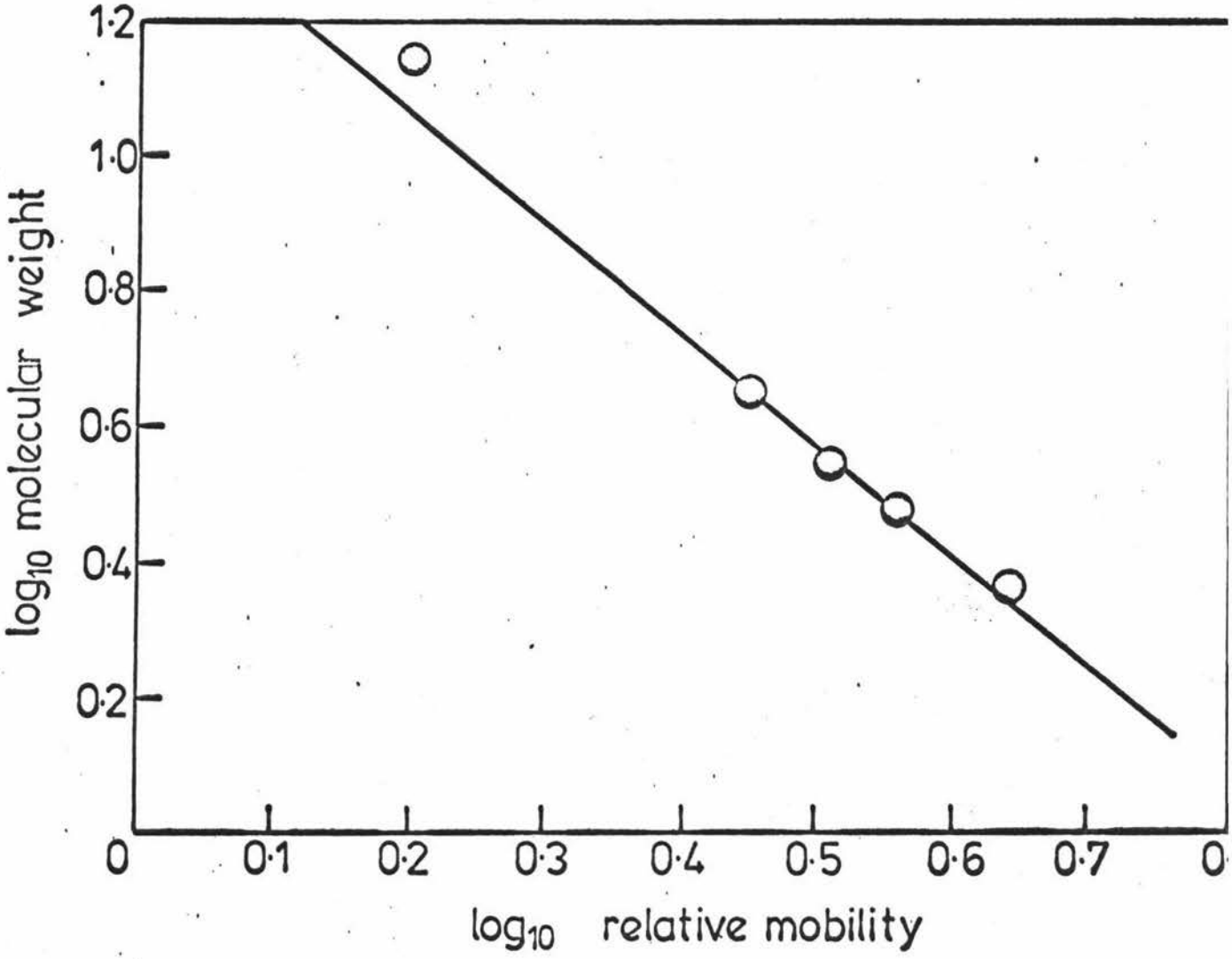


TABLE AIX: The relative mobilities and estimated molecular weights of fragments obtained by digesting plasmids from *Rhizobium phaseoli* NZP 5492 B5/8 and NZP 5492 B5/1 with *Eco* RI (plate 49)

relative mobility of fragment	$\log_{10}$ relative mobility	$\log_{10}$ estimated molecular weight	estimated fragment molecular weight (Md)
Rhizobium phaseoli NZP 5492 B5/8; 13 fragments ; estimated molecular weight 66.7Md			
1.64	0.21	1.04	11.0
1.87	0.27	0.95	8.9
2.17	0.34	0.84	6.9
2.27	0.36	0.80	6.3
2.42	0.38	0.77	5.9
2.57	0.41	0.72	5.2
2.68	0.43	0.69	4.9
2.80	0.45	0.65	4.5
2.98	0.47	0.62	4.2
3.66	0.56	0.48	3.0
4.27	0.63	0.36	2.3
4.72	0.67	0.29	1.9
5.04	0.70	0.24	1.7
Rhizobium phaseoli NZP 5492 B5/1; 14 fragments, estimated molecular weight 68.1Md			
1.41	0.15	1.14	13.8
1.80	0.26	0.96	9.0
2.13	0.33	0.85	7.1
2.25	0.35	0.82	6.6
2.54	0.40	0.74	5.5
2.68	0.43	0.69	4.9
2.87	0.46	0.64	4.4
3.38	0.53	0.52	3.3
3.52	0.55	0.49	3.1
3.99	0.60	0.41	2.6
4.32	0.64	0.34	2.2
4.53	0.66	0.31	2.0
4.79	0.68	0.28	1.9
5.02	0.70	0.24	1.7

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