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DEVELOPMENT OF A DNA HYBRIDISATION METHOD FOR THE IDENTIFICATION OF *RHIZOBIUM* AND *BRADYRHIZOBIUM*.

A Thesis Presented in Partial Fulfilment
of the Requirements for the Degree
of Master of Science in Microbiology
at Massey University, New Zealand

JEAN HEATHER GARMAN

1990

ABSTRACT

The potential of a DNA hybridisation method, utilising a biotin-labelling system with a streptavidin/alkaline phosphatase detection system (ENZO Biochem), was investigated as an identification method for *Rhizobium* species and *Bradyrhizobium* sp. *(Lotus)* strains using nodule, colony and pure DNA.

The method used for extracting DNA from colonies and crushed nodules and binding it to nitrocellulose did not purify the DNA sufficiently to stop non-specific binding occurring between the streptavidin-alkaline phosphatase conjugate and the sample. An alternative method of colony hybridisation that removed more of the cellular constituents was required. Only pure DNA could be used.

The method was altered as follows: i) Tris/EDTA buffer was used to terminate the colour reaction in place of allowing the membrane to air dry; ii) 5% milk powder was used in place of 10% bovine serum albumin in the blocking buffer, complex detection buffer and washing buffer used in the detection of hybridised biotin-labelled DNA; iii) 5% dextran sulphate was included in the hybridisation buffer to decrease the minimum hybridisation time from 6hr to 3hr.

Investigation of the effect of variable conditions on the intensity of colour produced showed that: i) the incubation of alkaline phosphatase with its substrate at room temperature resulted in fluctuation of the development time as the enzyme reaction rate is sensitive over this range of temperature (approximately $15^{o}C$ to $30^{o}C$); ii) increasing the concentration of labelled DNA in the hybridisation buffer increased the intensity of colour produced, the minimum concentration that could be used without lowering the detection limit was 200 ng/ml; iii) continued incubation of alkaline phosphatase with its substrate after colour development in the negative control had begun gave an increased colour intensity in the sample but since this increase was not proportional to that of the negative control the net response (sample minus control) decreased.

When genomic probes were hybridised with slot-blots containing homologous DNA the detection limit was between 63 and 125 ng of DNA. Both ^{32}P-labelled and biotin-labelled genomic *Rhizobium leguminosarum* biovar *trifolii* DNA probes were able to distinguish between *Rhizobium leguminosarum* and other *Rhizobium species* but not between the biovars of *R.leguminosarum*. To distinguish between closely related species or strains when using ^{32}P-labelled or biotin-labelled probes a specific DNA sequence was required for use as the probe.

Two distinct DNA homology groups have been described in *Bradyrhizobium* sp. *(Lotus)*. From a gene library of *Bradyrhizobium* sp. *(Lotus)* strain cc814S (homology group I) 8 clones were isolated that contained sequences that distinguish a representative of homology group I (strain cc814S) from a representative of homology group II (strain NZP2076). This was achieved by hybridising total genomic DNA from strain cc814S with total genomic DNA from strain NZP2076 and removing the single stranded specific sequences with hydroxylapatite. The specific DNA was used to probe the gene library. Increased selection for group-specific sequences by substituting another homology group I strain (NZP2021) for strain cc814S and subcloning one of the clones isolated gave inconclusive results but indicated that a group specific sequence could be derived in this way.

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INTRODUCTION

1.1 THE ROLE OF *RHIZOBIUM* IN BIOLOGICAL NITROGEN FIXATION

Plants require nitrogen for growth but are unable to assimilate atmospheric nitrogen. Chemical production of fertilizers that contain nitrogen that plants can assimilate uses non-renewable resources, is energy expensive and requires application of the fertilizer to the plants. An alternative source of utilizable nitrogen is atmospheric nitrogen which is fixed biologically by a wide range of bacteria. These may be free-living in the soil, part of the rhizosphere or involved in symbiotic associations with plants (Dixon, 1986).

Bacteria of the genus *Rhizobium* form symbiotic associations with legumes by nodulating their roots (Jordan, 1974). The significance of this genus is shown by the estimation that 50 - 70% of world biological nitrogen fixation is carried out by *Rhizobium*-legume associations (Quispel, 1974). This is important in countries, like New Zealand, with an agriculturally based economy where it has been estimated that 97% of the fixed nitrogen used annually is produced by the *Rhizobium*-legume association (MacKinnon, 1977). There is also potential in underdeveloped countries for the use of symbiotic nitrogen fixation to improve soil fertility and plant production and hence animal and human nutrition without incurring the costs and problems associated with the use of nitrogenous fertilizers. Therefore any improvement in the efficiency of the *Rhizobium*-legume association would be beneficial to the New Zealand community and to humanity generally.

Different species of *Rhizobium* have different nitrogen fixation efficiencies when nodulating the same species of legume. One legume species may be nodulated by several species of *Rhizobium* or by different strains of a species (Wilson, 1944; Graham, 1964). It is often desirable to know which species or strain is nodulating a plant. This is particularly relevant in the case of legume seeds that have been coated with a specific rhizobial innoculum strain before planting (for use in soils where either because of acid conditions or lack of resident legumes there are few rhizobia present or the strains present have a low nitrogen fixation efficiency) to determine if it is the innoculum strain or a soil strain that succeeds in nodulating the plant.

1.2 *LOTUS* SPECIES GROWN IN NEW ZEALAND AND THE *RHIZOBIUM* THAT NODULATE THEM

There are five main species of *Lotus* in New Zealand - the annuals *L. angustissimus* and *L. subbiflorus* and the perennials *L. tenuis, L.corniculatus* and *L. pedunculatus* with *L. pedunculatus* cav 'Grasslands Maku' being the most commonly sown cultivar. *Lotus* species give a greater yield than and show superior persistence to red or white clover and lucerne under low fertility conditions, low levels of nutrients (especially phosphate and potassium),high water table, poor drainage and soil salinity (Langer, 1973).

Rhizobium that nodulate *Lotus* species include both fast and slow-growing strains (Greenwood, 1977; Pankhurst, 1977). The fast-growing strains are classified as *Rhizobium loti* (Jarvis, 1982) while slow-growing strains are classified as *Bradyrhizobium* sp. *(Lotus)* (Jordan, 1982). Most *R. loti* strains form effective nodules on *L. tenuis* and *L.corniculatus* and ineffective nodules on *L. pedunculatus*, however, some strains can form effective nodules on all three. *Bradyrhizobium* sp. *(Lotus)* only form effective nodules on *L. pedunculatus*. The slow-growing strains can be divided into two distinct DNA homology groups as determined by DNA reassociation experiments (Chua, 1984) (Figure I).

1.3 REQUIREMENTS OF AN IDENTIFICATION METHOD

Bacterial identification methods are used to detect known strains after they have been added to the environment and to identify strains isolated from the environment.

The requirements for an identification method are:
1) that it is dependent on a unique characteristic of the organism to be identified.
2) that the characteristic is stable in that organism.
3) that results can be obtained quickly.
4) that the results are reproducible.
5) that the procedure is simple and requires minimum effort or technical expertise.
6) that the method is economical to set up.
7) that the method is economical to run.

Although some of these characteristics will be mutually exclusive for many methods, the ideal method for a particular application will be one that fulfils the main requirements of the application with as many other characteristics as possible.

1.4 DNA HYBRIDISATION AS AN IDENTIFICATION METHOD

DNA hybridisation can be defined as the annealing of two complementary strands of nucleic acid molecules. Due to the specific interaction between complementary base pairs a single stranded DNA sequence that has been complexed with a detectable label can be used to locate a complementary sequence (Enns, 1988). DNA hybridisation can be carried out on samples that have been immobilised on a membrane. Either crude cell extracts (*eg* colony blots) or purified total genomic DNA (*eg* slot-blots) or restriction endonuclease digests (*eg* Southern blots) can be used. The probe can be made from either total genomic DNA or a specific sequence of DNA or rRNA.

DNA hybridisation fulfils most of the requirements for an identification method. As a species often has little homology with related species it follows that a portion of the genome is unique to that species. Every organism must have a complete genome to be viable and DNA does not undergo change from generation to generation (except for very small rearrangements, larger rearrangements usually result in a non-viable organism), therefore the genomic DNA is a stable characteristic of a species. However, DNA carried on plasmids is not necessarily stable as it is more readily lost or rearranged. Different hybridisation methods take different periods of time to obtain results depending on the sample being tested, whether the sample is probed directly or the DNA is extracted and purified, and on the labelling and detection systems used. As the DNA-DNA interaction is highly specific and sensitive the results are usually reproducible. The simplicity and the cost varies from system to system.

DNA hybridisation has several advantages over other systems. These include the ability to detect both expressed and unexpressed genes whereas other methods are only able to detect expressed genes. DNA hybridisation can be performed directly on a sample where as most other methods require culturing of the organism before a test can be carried out. The results can be read objectively by instruments and so need not

be interpreted subjectively. These factors combined with high levels of specificity and sensitivity (over which control can be exerted by altering the temperature and salt concentration of the hybridisation reaction) this leads to more accurate results being produced.

1.5 NON-RADIOACTIVE HYBRIDISATION SYSTEMS

A label is a molecule that is bound to the probe DNA which can be detected after the probe DNA has been hybridised to the target DNA. The requirements of a label for a DNA probe are:

1) that it is easily attached to DNA.
2) that it is detectable at very low concentrations using simple instrumentation.
3) that it is stable at the elevated temperatures required to achieve specificity in the hybridisation method.

1.5.1 TYPES OF NON-RADIOACTIVE DETECTION SYSTEMS

There are four types of non-radioactive detection systems that can either be used to label DNA or RNA or to detect labelled DNA or RNA when combined with an affinity system to the label. The detection systems are:

1) **Chemiluminescent** *eg* isoluminol. Detected by exposure to X-ray film or with a luminometer (Urdea, 1988; Gillam, 1987),
2) **Electron Dense** *eg* ferritin, electron dense proteins, colloidal gold. Detected by electron microscopy (Richardson, 1983; Pereira, 1986),
3) **Fluorescent** *eg* fluorescien, Texas red, rhodamine, isothiocyanate. Detected by emission spectra (spectrofluorimeter) (Urdea, 1988; Bauman, 1981),
4) **Enzymatic** *eg* alkaline phosphatase, acid phosphatase, horse radish peroxidase. Detected by incubation with a chromogenic substrate which results in deposition of pigment in the vicinity of the enzyme. (Urdea, 1988; Zwadyk, 1986).

The limit of detection for a hybridisation system depends on the system used. Molecules which amplify the signal by giving several signals per molecule (*eg* enzymes) are likely to be more sensitive than molecules that give a single signal per molecule (*eg* fluorscein)

Although all four types of detection system have their applications, enzymatic systems have been used most. It has been suggested that horseradish peroxidase and alkaline phosphatase are superior to fluorescent or chemiluminescent detection systems (Urdea, 1988) while electron dense systems have limited use as they require special equipment and expertise for detection. It has been reported (Gillam, 1987), that peroxidase enzymes have certain disadvantages compared to phosphatase enzymes in that i) some of the substrates are known carcinogens, ii) they give dull colours that are hard to visualise and photograph, iii) the substrates slowly form pigment in the absence of peroxidase, limiting the incubation time before the background becomes unacceptable and iv) many tissues naturally contain peroxidases.

1.5.2 LABELLING SYSTEMS THAT UTILISE EITHER DIRECT DETECTION OR AN AFFINITY SYSTEM FOR DETECTION

Labelled DNA can be detected either directly if the label can be visualised or by means of a detectable affinity system if the label itself cannot be visualised.

Labelling systems for DNA and RNA that can be detected directly include:

1) the joining of a fluorescent, chemiluminescent or enzyme label onto an oligonucleotide after deprotection and purification (Urdea, 1988).
2) covalently linking an enzyme, that has been crosslinked to polyethyleninime using p-benzoquinone, to single stranded DNA with gluteraldehyde (Renz, 1984).
3) coupling fluorochromes to the oxidised 3' terminus of RNA with hydrazine in a mixture of dimethylsulfoxide and pyridine (Bauman, 1981)
4) transferring the non-adenyl portion of derivatives of p^1-(6-aminohex-1-yl)-p^2-(5'adenosine) pyrophosphate containing fluorescien or tetramethylrhodamine with T4 RNA ligase to the 3-hydroxyl terminus of RNA (Richardson, 1983),
5) covalently linking ferritin to RNA (Wu, 1973).

Affinity systems are designed to increase sensitivity by using detection systems that are not directly linked to the probe so can give an amplified signal by binding more than one detection molecule per label molecule (Renz, 1984). There are two main types of affinity systems, those involving antibodies and those involving the biotin and avidin affinity.

Antibodies tagged with a detectable molecule *eg* an enzyme, colloidal gold or a fluorescent compound that are specific for:

1) double stranded polynucleotide co-polymers complexed with methylated serum albumin (detects double stranded RNA) (Stollar, 1970),
2) ribosomal RNA genes where the rRNA has bound to the DNA (Rudkin, 1977),
3) an antigen that has been attached to a base used in the synthesis of the probe DNA (*eg* antibodies specific to nucleic acid adducts which are formed by modification of nucleic acids with N-aceloxy-N-2-acetylaminofluorine or by its iodinated derivative) (Tchen, 1984).

A majority of systems function around the incorporation of biotin into the probe DNA because of its high affinity for avidin ($K = 10^{15}$ per mol) and its minimal interference with hybridisation (Chan, 1985). The biotin-labelling system coupled with the avidin/enzyme system of detection has been reported as being 20 - 50 times more sensitive than immunological techniques (Leary, 1983).

Biotin can be attached directly to the probe DNA by:

1) transaminating the unpaired cytosine residues with ethylenediamine and sodium bisulfate, the primary group on the derived cytosine is then reacted with biotinyl-e-aminocaproic acid N-hydrosuccinimide ester (Viscidi, 1986)
2) covalently linking a biotin molecule to RNA or DNA through di-imide (Broker, 1978), cytochrome C (Manning, 1975) or histone (Renz, 1983) bridges.
3) end labelling oligonucleotides (15 to 40 base pairs long) with a aminoalkylphosphoramine linker arm on the 5'-termini (Chollet, 1985).
4) using photobiotin (N-(4-azido-2-nitrophenyl)-N'-(Nd-biotinyl-3-aminopropyl)-N'-(methyl-1,3-propenediamie)) which will form a stable link with nucleic acids after a brief irradiation with visible light (Forster, 1985).

Biotin can be attached to modified nucleotides that can be subsequently incorporated into the DNA by oligonucleotide synthesis, complementary strand synthesis (nick translation, random primer extension), 3' end labelling or by filling in the cohesive ends created by digestion with restriction endonucleases. The modification of dUTP is carried out by the attachment of a biotin molecule via linker arms 4, 7, 11, 16 or 21 atoms long to the C5 pyrimadine ring (Leary, 1983). Other nucleotides have also been used, attaching biotin via aliphatic linkers 3-17 atoms long to the H-bonding positions 6N on adenine and 4N on cytosine (Gebeyehu, 1987). These modified nucleotides are referred to as Bio-n-dNTP where n is the number of atoms in the linker.

The level of incorporation into a DNA strand of biotin modified nucleotides varies according to the length of the linker arm, since the biotin molecule is relatively large if the linker arm is short there is less incorporation as there is less room for the biotin molecules (Gebeyehu, 1987). At high levels of substitution the melting temperature is lowered (Langer, 1981; Leary, 1983) and the Cot1/2 for reassociation is five times greater than unsubstituted DNA (Gebeyehu, 1987). However, levels of substitution of biotin beyond 7 - 31 biotin molecules per kilobase are not necessary as above this level there is no increase in sensitivity (Gebeyehu, 1987) and at these low levels of biotin substitution (anything less than 150 molecules per kilobase) the denaturation, reassociation and hybridisation characteristics are similar to unsubstituted DNA (Langer, 1981).

The biotin label is detected by incubation with avidin or streptavidin coupled to a detection system. Streptavidin, although having the same affinity as avidin, has been reported as giving less non-specific binding due to its iso-electric point being closer to neutrality than that of avidin (the iso-electric point of avidin is 10, so at neutral pHs it is positively charged) and due to streptavidin containing no carbohydrate compared with avidin which contains 7% carbohydrate (manufacturers information).

1.5.3 ADVANTAGES OF NON-RADIOACTIVE HYBRIDISATION SYSTEMS

Recently there has been an increasing interest in non-radioactive probes which have several advantages over their radioactive counterparts. These advantages include:

1) **Stability** - while ^{32}P has a half life of fourteen days non-radioactive probes have remained functional for up to 2 years.
2) **High Resolution** - the pigment produced is deposited only where the enzyme is found whereas the radiation emitted by ^{32}P tends to give a diffuse mark on the film emulsion used for detection.
3) **Safety** - there are no inherent health risks as with radioactivity so special handling and disposal procedures are not required, therefore there is only a modest requirement for laboratory facilities.
4) **Simple and Rapid Detection** - there is no requirement for long auto-radiograph exposures with reported detection times for non-radioactive probes ranging from 20 minutes (Renz, 1984) to 24 hours (Leary, 1983) depending on the system and application used.
5) **Specificity** - there is less non-specific binding of non-radioactive probes to nitrocellulose at high probe concentrations (250 - 750ng/ml) (Leary, 1983).

6) **Versatility** - more than one probe can be sequentially used per membrane if different detection systems are used (*eg* to define orientation of large genes or to detect different viruses in the same sample) (Renz, 1984).

A time-cost analysis (Zwadyk, 1986) shows that the biotin-labelling systems are quicker than ^{32}P-labelling systems but that there is little difference in cost (Table I).

The main disadvantage reported with non-radioactive labelling systems is a decrease in sensitivity (although this is not consistent) when compared to radioactive labelling systems. The reported limits of detection show considerable variation (Table II). It has also been reported that biotin-labelled probes have decreased efficiency of hybridisation requiring a higher probe concentration (approximately 50 - 100ng/ml) than a radioactive probe to achieve the same result (Gebeyehu, 1987).

1.6 ISOLATION OF SPECIFIC DNA SEQUENCES FOR USE AS PROBES

Radioactive or non-radioactive DNA hybridisation with genomic DNA probes can be used to distinguish between species that are not closely related (Cooper, 1987; Hodgson, 1983; Imaeda, 1988). To distinguish between closely related species or strains a specific DNA sequence is required for use as the probe since total genomic DNA probes show too much cross-reaction for differentiation (Cooper, 1987; Hodgson, 1983; Welcher, 1986).

There are four basic procedures for acquiring a specific DNA sequence for use as an identifying probe.

1) A known strain or species-specific gene is used as a probe. For example: a piece of the *npt*II gene from a locus essential for formate-dependent growth of *Bradyrhizobium japonicum* was used as a specific probe for *B. japonicum* in soil (Holben, 1988).
2) Random DNA fragments are cloned and screened with genomic probes from the species to be distinguished between *eg* to obtain sequences specific for clinically important *Bacteroides* species which have cross-homologies of 14 to 28% (Saylers, 1983; Kurtiza 1985, 1986).

TABLE I : Time/cost Analysis of Hybridisation Systems with 100cm2 Filters (96 samples) (Zwadyk, 1986)

		BIOTIN-STREPTAVIDIN	
	^{32}P	BRL alkaline phosphatase	ENZO Biochem acid phosphatase
Total time	47.5hr	27hr	27hr
Hands on time	60min	54min	41min
Labour cost[a]	$10.48	$9.45	$7.18
Kit cost		$2.75	$0.90
Other reagents	$1.60	$3.15	$4.33
Probe	$3.15	$1.25	$1.25
Total	$15.23	$16.60	$14.66

a = Salary of G5-9, Step 1, Medical Technologist, $10.48/hr

TABLE II : Reported Detection Limits for Biotin Probe Systems

DETECTION SYSTEM	LIMIT OF DETECTION	REFERENCE
^{32}P	5.2 ng	Matthews, 1988
^{32}P	10 - 12.5 ng	Zwadyk, 1986
B/S/AP	0.2 - 4.0 ng	Matthews, 1988
B/S/AP, B/S/AcP	22 ng	Zwadyk, 1986
B/S/AP, B/S/AcP	1 ng	Amersham
B/S/AP	1 ng	Urdea, 1985
B/S/AP, B/S/AcP	20 - 50 pg	Zeph, 1989
B/S/AP	5 - 10 pg	Renz, 1984
B/S/AP	1 - 10 pg	Leary, 1983
B/S/AP	0.25 pg	Gebeyuhu, 1987
B/S/AP, B/S/AcP	0.1 pg	Chan, 1985

B/S/AP = biotin-streptavidin/alkaline phosphatase detection system
B/S/AcP = biotin-streptavidin/acid phosphatase detection system

3) A gene library of an organism is probed with related organisms to locate specific fragments. For example; specific *Campylobacter jejuni* sequences were found by screening a gene library with *C. jejuni* and *C. coli*, and picking the plaques that reacted to the former but not the later for further screening against other *Campylobacter* species (Kurtza, 1986).

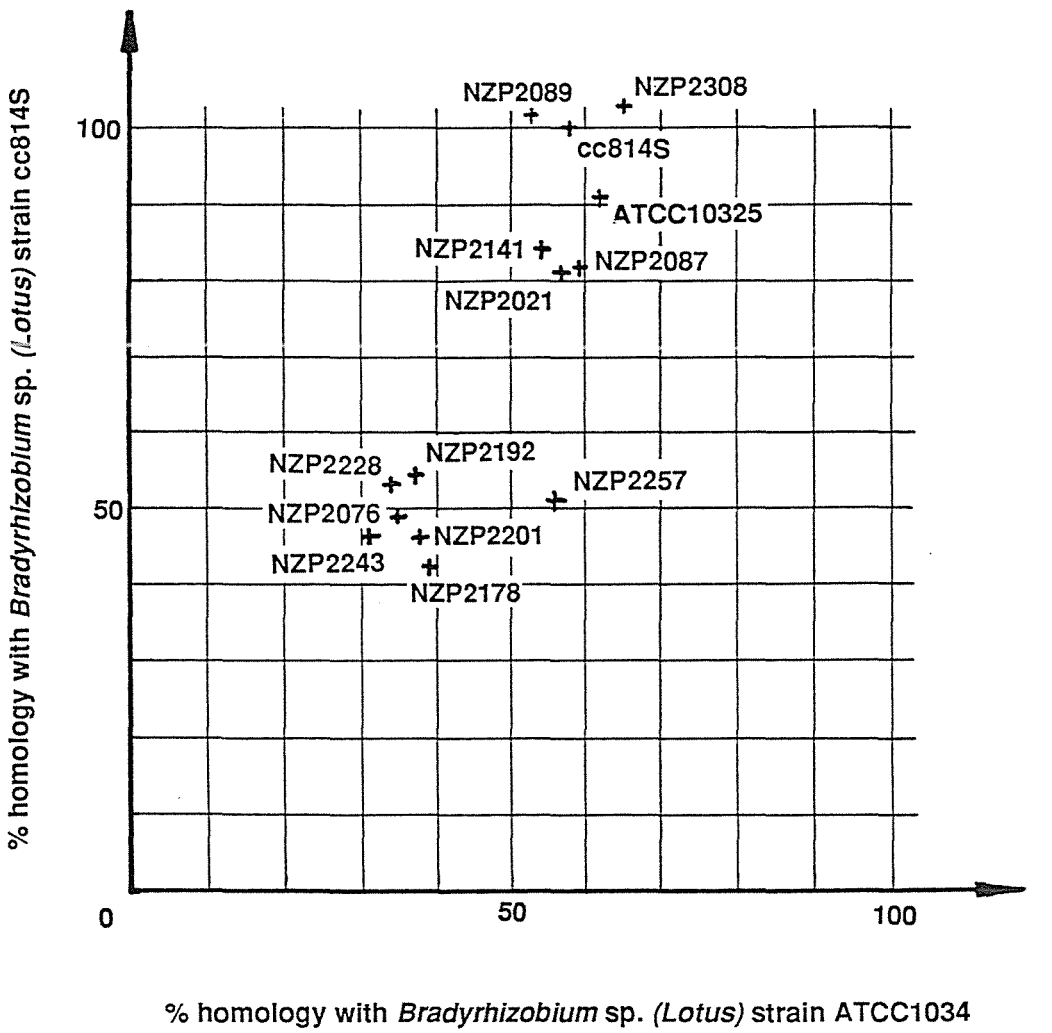
4) Selection of specific sequences to increase the chance of detecting specific clones in a gene library. For example:
   i) a set of bands, from restriction endonuclease digests, specific for an organism may be cloned and used to identify highly abundant genomic sequences from a gene library (Garfinkel, 1989).
   ii) specific sequences that relate to an expressed surface gene eg membrane proteins and antigens can be isolated from a gene library, that expresses the insert sequences, with polyclonal antisera (Korolik, 1988).
   iii) DNA from other strains/species can be used in positive selection procedures for the enrichment of specific sequences by hybridisation of non-specific sequences to subtracter DNA which is immobilised on a solid support (agarose, dextran, cellulose or nitrocellulose) *eg* i) specific sequences for *Rhizobium loti* were isolated by hybridising radio-labelled target DNA to a subtracter DNA-cellulose complex in solution. The specific sequences remain in solution and can be removed from the non-specific sequences that have bound to the subtracter DNA-cellulose complex. The matrix can then be washed to remove the non-specific DNA so that it can be used again (Cooper, 1987).

1.7 AIMS OF THIS INVESTIGATION

1. To modify a standard biotin labelling, streptavidin-alkaline phosphatase detection system for use as an identification method for *Rhizobium* and *Bradyrhizobium* species.

2. To isolate a specific DNA sequence capable of distinguishing between the two homology groups of *Bradyrhizobium* sp. *(Lotus)*.

Figure 1: The two homology groups of *Bradyrhizobium* sp. *(Lotus)* as determined with hydroxylapatite batch processing after hybridisation at 65^{o}C for 40hr in 0.28M phosphate buffer pH6.8 (Chua, 1984).


NZP2089
NZP2308
cc814S
ATCC10325
NZP2141
NZP2087
NZP2021
NZP2192
NZP2228
NZP2257
NZP2076
NZP2201
NZP2243
NZP2178
100
50
0
50
100
% homology with *Bradyrhizobium* sp. *(Lotus)* strain cc814S
% homology with *Bradyrhizobium* sp. *(Lotus)* strain ATCC1034

MATERIALS AND METHODS

All media and solutions were made up to volume with deionised water unless otherwise stated and all chemicals used were AR grade or equivalent.

2.1 Media

All media was sterilized at 121°C for 15 min.

Yeast Mannitol Agar (YMA) (Vincent, 1970) contained (g/l): mannitol (BDH), 10.0; yeast extract (Difco), 5.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; agar (Davis), 15.0. The pH was not adjusted. Congo red dye (0.0025%) was added to facilitate the detection of contaminant bacteria (Hahn, 1966).

Brain Heart Infusion Agar (BHI) contained (g/l): brain heart infusate (Difco), 37.0; agar (Davis), 15.0.

Tryptone Yeast Extract Broth (TY) (Beringer, 1974) contained (g/l): tryptone (Difco), 5.0; yeast extract (Difco), 3.0; $CaCl_2.6H_2O$, 1.3 (autoclaved separately as a 1M solution and added to the TY broth after it had been autoclaved to avoid the formation of a precipitate). TY agar was obtained by adding 15.0g of agar (Davis) to each litre of this medium.

Water Agar contained 15.0g of agar (Davis) per litre of deionised water.

Thornton's Seedling Agar (Thornton, 1930) contained (g/l): $Ca_3(PO_4)_2$, 2.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $FePO_4$, 1.0; $FeCl_3.6H_2O$, 0.017; agar (Davis), 15.0; and 1ml of Hoglands Trace Element Solution. This agar was dispensed in 10ml aliquots into 15 x 150 mm test tubes with caps before autoclaving and allowed to set on a slant.

Hoglands Trace Element Solution contained (g/l): H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.H_2O$, 0.22; $CuSO_4.H_2O$, 0.08; $CuSO_4.7H_2O$, 0.095; $Na_2MoO_4.2H_2O$, 0.054. The solution was dissolved by heating.

Luria Broth (LB) (Millar, 1972) contained (g/l): NaCl, 5.0; tryptone, 10.0; yeast extract (Difco), 5.0. LB agar was obtained by adding 15.0g of agar (Davis) to each litre of this medium. LB containing tetracycline (LB^{TC}) was obtained by adding 15mg of tetracycline (dissolved in 5mls of methanol) to the media after it was autoclaved. LB containing ampicillin (LB^{AMP}) was obtained by adding 75mg of ampicillin (dissolved in deionised water and filter sterilised) to the media after it was autoclaved.

2.2 Bacterial Strains and Maintainence

The bacterial strains used in this investigation were obtained from the Microbiology and Genetics Department, Massey University, freeze dried collection and are listed in Table III. Rhizobia were isolated and cultivated on Yeast Mannitol Agar (YMA) at 28°C. All stock cultures were streaked on YMA and BHI to check for purity. Only single well isolated colonies were used for subsequent inoculations. All *Rhizobium* strains except *Bradyrhizobium* sp.(*Lotus*) were checked for their ability to nodulate the appropriate species of plant. All *Rhizobium* strains were maintained on YMA slopes at 4°C and subcultured at intervals of 4-6 months. The *Escherichia coli* strain was grown and maintained on BHI. All strains were also stored in 50% glycerol at -16°C.

2.3 Plant Inoculation

Surface Sterilisation of Seeds. Legume seeds were scarified (by briefly rubbing them between two sheets of emery paper) placed in a glass cylinder with nylon mesh over one end, immersed momentarily in 95% ethanol and then in a freshly prepared 1% sodium hypochlorite solution for 10min. This was followed by thorough washing in five changes of sterile water. The mesh was slit with a sterile scalpel blade and the seeds placed in a sterile petri dish. Seeds were transferred onto the surface of a Water Agar plate with a sterile loop and incubated at room temperature in the dark for 2-3 days.

Seedling Inoculation and Growth. A germinated seedling was transferred aseptically from the Water Agar plate to a tube of Thornton's Seedling Agar. 100ul of

TABLE III : The Bacterial Strains used in this Study and the Plants they Nodulate

SPECIES	STRAIN	PLANT ORIGIN
Rhizobium leguminosarum bv. *trifolii*	TA1	*Trifolium repens*
Rhizobium leguminosarum bv. *trifolii*	ICMP2668	*Trifolium repens*
Rhizobium leguminosarum bv. *trifolii*	1/6	*Trifolium repens*
Rhizobium leguminosarum bv. *trifolii*	505/1 = TLN3	*Trifolium repens*
Rhizobium sp. *(Galega)*	Gal 1	*Galega officialis*
Rhizobium sp. *(Galega)*	Gal 14	*Galega officialis*
Rhizobium fredii	USDA 191	*Glycine max*
Rhizobium fredii	USDA 208	*Glycine max*
Rhizobium leguminosarum bv. *leguminosarum*	NZP5224	*Phaseolus vulgaris*
Rhizobium leguminosarum bv. *phaseoli*	NZP5232	*Phaseolus vulgaris*
Bradyrhizobium japonicum	NZP5531	*Glycine max*
Bradyrhizobium japonicum	NZP5536	*Glycine max*
Rhizobium meliloti	U45	*Medicago sativa*
Rhizobium meliloti	NZP4009	*Medicago sativa*
Bradyrhizobium sp. *(Lotus)*	NZP2076	*Lotus hispidus*
Bradyrhizobium sp. *(Lotus)*	NZP2257	*Lotus coniculatus*
Bradyrhizobium sp. *(Lotus)*	NZP2192	*Lotus coniculatus*
Bradyrhizobium sp. *(Lotus)*	NZP2228	*Lotus angustissimus*
Bradyrhizobium sp. *(Lotus)*	NZP2307 = cc814S	*Lotus pedunculatus*
Bradyrhizobium sp. *(Lotus)*	NZP2089	*Lotus pedunculatus*
Bradyrhizobium sp. *(Lotus)*	NZP2021	*Lotus pedunculatus*
Escherichia coli	B113	
Escherichia coli	HB101	

a turbid suspension of the inoculant was added to the plant in the tube. The tubes were incubated at 22oC with 16hr of light and 8hr of dark per day.

Isolation of Bacteria from Nodules. When nodules began to form (approximately 3 weeks) they were removed from the root, placed in a glass cylinder with nylon mesh over one end, momentarily immersed in 95% ethanol and then in a 5% hydrogen peroxide solution for 10min. This was followed by thorough washing in five changes of sterile water. The mesh was slit with a sterile scalpel blade and the nodules placed in a sterile petri dish. To each nodule 100ul of sterile water was added and the nodule was crushed with a sterile glass rod. The resulting emulsion was plated out on YMA and incubated at 28^{o}C until colonies had formed.

2.4 Extraction of Bacterial DNA

2.4.1 Materials

Tris EDTA buffer (TE buffer) contained 10mM Tris (Tris[hydroxymethyl]aminomethane) and 1mM EDTA (Ethylene diamine tetra acetic acid). The pH was adjusted to 7.5 with hydrochloric acid (HCl).

Lysozyme stock solution contained Lysozyme (BDH) at a concentration of 10 mg/ml. It was stored at -20^{o}C.

Protease type XIV stock solution contained Protease type XIV (Sigma) at a concentration of 20 mg/ml. It was incubated at 37^{o}C for 2hr to destroy nuclease activity and stored at -20^{o}C.

Ribonuclease stock solution contained Ribonuclease (Sigma) at a concentration of 10 mg/ml. It was boiled for 15min to destroy nuclease activity and stored at -20^{o}C.

Sodium Lauryl Sulphate (SLS) stock solution was 25% SLS.

Sodium Perchlorate stock solution was 5M sodium perchlorate.

Dialysis Buffer contained 0.1M Tris, 1M NaCl and 80mM $MgCl_2.6H_2O$. The pH was adjusted to 7.5 with HCl. It was stored at 4^{o}C and diluted tenfold before use.

Redistillation of Phenol. The glass distillation apparatus (Figure 2) was constructed from standard 'Quickfit' fittings. Liquid phenol (1.5kg) was placed in flask A along with 1% w/v aluminium turnings and 0.05% w/v sodium bicarbonate and heated to 100°C. Water and other volatile impurities in the sample were boiled off. Once these had been removed and the temperature rose, a vacuum of 100mm of Hg (obtained by adjusting tap E) was applied by opening tap D when the temperature reached 174°C. Under these conditions phenol vaporised, condensed in tube B and collected in flask C. The redistilled phenol was stored frozen in sealed dark containers at -16°C.

Phenol/Chloroform/Iso-amyl alcohol was prepared by mixing redistilled phenol (melted at 50°C) with an equal volume of chloroform. Iso-amyl alcohol was added to give a final concentration of 4%. TE buffer was added until two phases were apparent and the mixture was stored in a dark container at 4°C.

Dialysis Tubing was prepared by cutting cellulose dialysis tubing (Union Carbide) into lengths and boiling it for 10min in several changes of deionised water. It was stored at 4°C in deionised water.

2.4.2 Method

Tryptone Yeast Extract broth (25ml) was inoculated from a well isolated colony of the required bacterial strain and incubated at 28°C until turbid. The broth culture was streaked on YMA and BHI agar to check for purity. The broth was centrifuged for 10min at 8 000g and the supernatant discarded. The pellet was resuspended in TE buffer (10ml), recentrifuged, resuspended in TE buffer (10ml) containing lysozyme (1 mg/ml) and incubated at 37°C for 1hr. Protease type XIV (0.5 mg/ml) was added and incubation at 37°C was continued for 1hr. Sodium Lauryl Sulphate (1%) and ribonuclease (50 ug/ml) were added and incubation at 37°C was continued overnight. The digest was made 1M with respect to sodium perchlorate and incubated at 37°C for 2hr. An equal volume of phenol/chloroform/iso-amyl alcohol was added, mixed by shaking for 20sec and centrifuged for 10min at 6 000g. The upper phase was removed to a clean centrifuge tube and the process repeated until no white precipitate formed at the interface. The upper phase was then placed in a dialysis bag and dialysed at 4°C against six changes of dialysis buffer (500ml) over a period of three days. The DNA solution was removed from the dialysis bag to a sterilised container and incubated at 65°C for 10min to destroy any nuclease activity.

Figure 2: The apparatus used for the distillation of phenol

A = flask where phenol to be distilled is placed

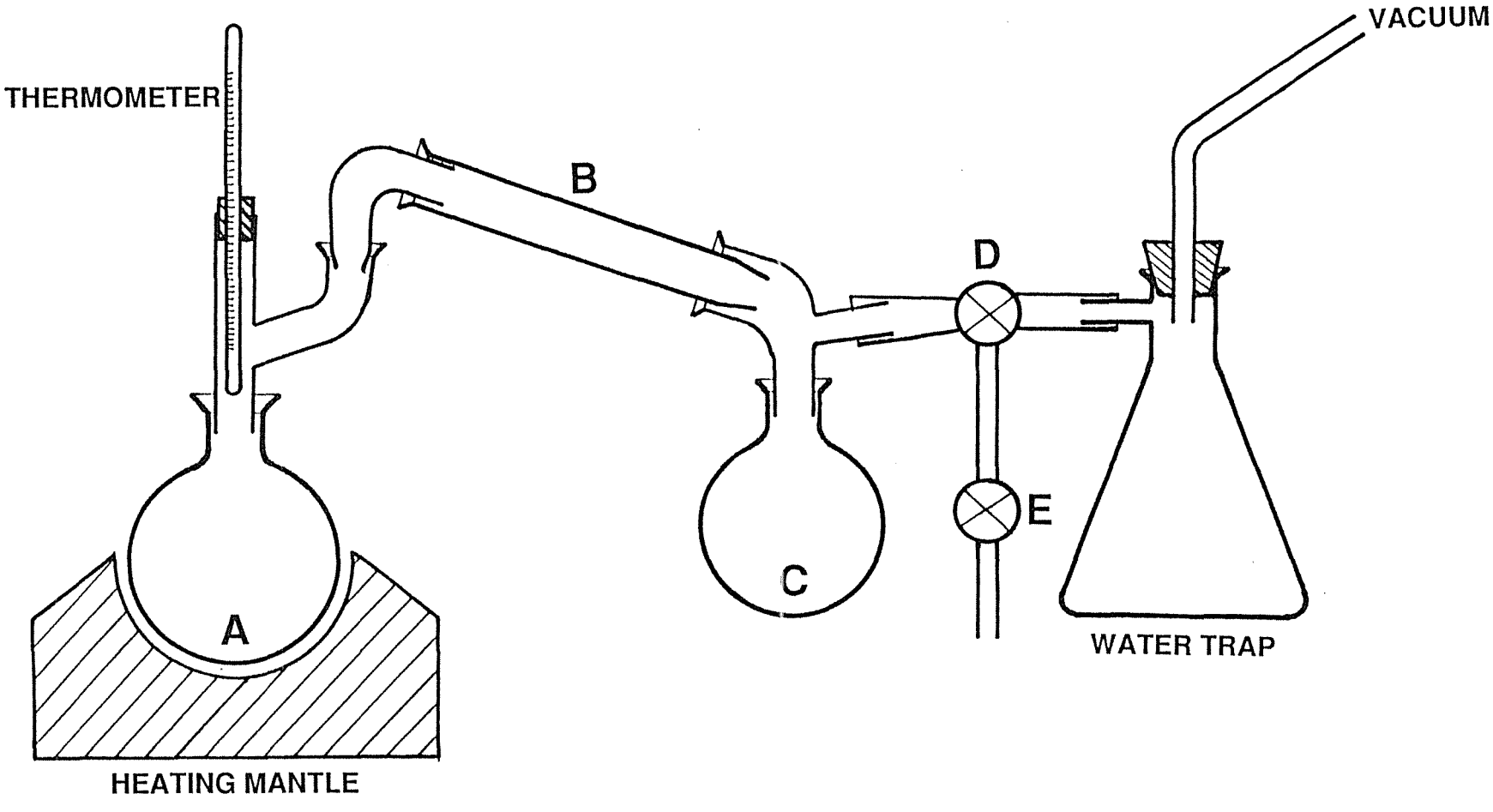
B = condenser tube

C = flask where distilled phenol is collected

D = tap used to apply the vacuum to the system

E = tap used to control vacuum

(see section 2.2.4 for details).


VACUUM
THERMOMETER
B
D
E
C
A
WATER TRAP
HEATING MANTLE

2.5 Determination of DNA Concentration and Purity

A sample of the DNA solution was diluted 1 in 20 and a blank was prepared from a 1 in 20 dilution of the dialysis buffer in water. Samples were scanned in a 1ml cell on an automatic scanning spectrophotometer (Cecil CE 599) at wavelengths from 220nm to 320nm. The DNA concentration was calculated from the formula

$$\text{DNA concentration (mg/ml)} = \frac{(OD258 - OD300) \times \text{dilution factor}}{20}$$

To determine whether all the protein had been removed the ratio of the OD_{258}:OD_{230} was calculated. A ratio greater than 1.8 was required. If the ratio was lower the sample required further phenol/chloroform/iso-amyl alcohol extractions.

To determine whether all the phenol had been removed the ratio of the OD_{258}:OD_{280} was calculated. A ratio greater than 1.8 was required. If the ratio was lower the sample required further dialysis or chloroform extraction.

2.6 Ethanol Precipitation of DNA

2.6.1 Materials

Sodium Acetate stock solution was 3M sodium acetate (NaAc).

Ethanol was 95% ethanol.

2.6.2 Method

To the DNA sample 1/10 of the volume 3M NaAc and 2.5 volumes of ethanol were added. The sample was mixed by inversion, held at -16^{o}C overnight and then centrifuged for 20min at 10 000g. The supernatant was discarded and the pellet dried by vacuum dessication for 15min before being resuspended in sterile water at the required concentration and stored at 4^{o}C.

2.7 Agarose Gel Electrophoresis

2.7.1 Materials

Electrophoresis Buffer contained 40mM Tris, 5mM NaAc and 1mM EDTA. The pH was adjusted to 7.8 with glacial acetic acid.

Dye Solution contained (g/100ml): sucrose, 50.0; SLS, 0.1; Bromophenol blue, 0.05.

Ethidium Bromide stock solution contained ethidium bromide at a concentration of 0.04 mg/ml.

Decolourising Solution was 1% $MgCl_2$.

2.7.2 Method

An 0.8% agarose gel was prepared by adding 0.2g of Agarose (Bio-Rad ultra pure DNA grade) to 25ml of electrophoresis buffer and refluxing the mixture until the agarose was completely dissolved (5-10min). The gel was poured into a 100 x 60 mm holder and allowed to set with a "Teflon" comb in place to create wells. Samples loaded into the wells contained 0.1 - 0.2 ug of DNA and 5ul of the dye solution in a total volume not exceeding 20ul. Electrophoresis was carried out in a horizontal electrophoresis apparatus (Bio-Rad Mini-Sub DNA cell) at 60 volts for 2hr. After electrophoresis the gel was submerged in ethidium bromide solution for 10min, and then in decolourising solution for 10min before being visualised on a UV transilluminator at 265nm and photographed on Polaroid 667 black and white film.

2.8 Preparation of Dot-Blots

2.8.1 Materials

Lysing Solution contained 1M NaOH and 3M NaCl.

Neutralising Buffer contained 0.5M Tris and 1.5M NaCl. The pH was adjusted to 7.5 with HCl.

Ethanol was 90% ethanol.

2.8.2 Method

A 15cm x 20cm piece of Whatman 3MM filter paper was soaked in approximately 30ml of neutralising buffer in a dish and a circular (8cm diameter) piece of nitrocellulose (Schleicher and Schuell BA 85, porosity 0.45 microns) was placed on top of the filter paper. DNA (0.5 mg/ml) was serially diluted in an equal volume of lysing solution, left to stand for 5min at room temperature, then 2ul volumes where placed on the surface of the nitrocellulose in a grid pattern and allowed to adsorb. The nitrocellulose was moved to a fresh area of the saturated filter paper, left to stand at room temperature for 5min, transferred to a filter holder and washed twice with 25ml of ethanol under vacuum. Once all the ethanol had been sucked through, the nitrocellulose was placed on a fresh piece of filter paper to dry and then baked for 2hr at 80^{o}C in a vacuum oven.

2.9 Preparation of Slot-Blots

2.9.1 Materials

Ammonium Acetate stock solution was 2M ammonium acetate. The pH was adjusted to 7.0 with HCl.

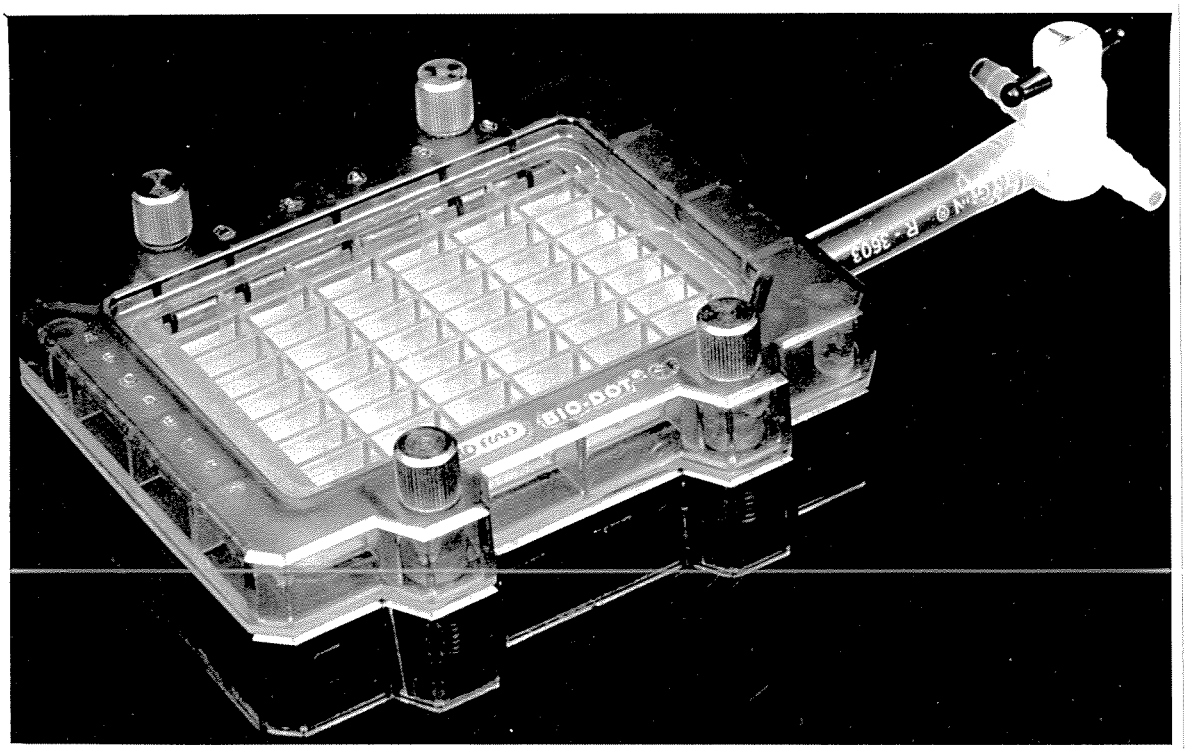
20X SSC contained 3M NaCl and 0.3M sodium citrate. The pH was adjusted to 7.0 with NaOH.

Sodium Hydroxide stock solution was 0.4M NaOH.

2.9.2 Method

3 sheets of Bio-Dot Slot Format Filter Paper and a piece of nitrocellulose cut to the same size were wetted in 6xSSC. They were placed in the Slot-Blot apparatus (Bio-Rad) (Figure 3) which was screwed together as tightly as possible. The required amount of DNA was denatured in 100ul of 0.4M NaOH for 5-10min, neutralised by the addition of 100ul of cold ammonium acetate solution and added to the wells under gentle vacuum (tap open). Once all the liquid had been drawn through (1-2hr) each

Figure 3: The slot-blot apparatus (Bio-Rad) used to apply DNA to nitrocellulose membranes (see section 2.2.9 for details).



slot was washed with 200ul of 2xSSC, which was drawn through under full vacuum (tap closed). The apparatus was disassembled and the nitrocellulose was rinsed in 2xSSC, placed on a piece of filter paper to dry and baked for 2hrs at 80^{o}C in a vacuum oven.

2.10 Preparation of DNA Random Primers (Jarvis, 1983)

2.10.1 Materials

Equilibration Buffer contained 5mM Tris, 1mM EDTA and 0.1M NaCl. The pH was adjusted to 7.4 with HCl.

Elution Buffer contained 5mM Tris, 1mM EDTA and 0.3M NaCl. The pH was adjusted 7.4 with HCl.

Ethanol was 95% ethanol.

Sodium Chloride stock solution was 0.2M NaCl.

2.10.2 Method

Calf Thymus (Sigma, 1g) was dissolved in 25ml of DNase buffer to a final concentration of 40 mg/ml. DNase 1 (deoxyribonuclease 5'-oligonucleotide hydrolase, 2mg) was added and the mixture was incubated at 37^{o}C for 45min. Following incubation the DNase 1 was destroyed by the addition of SLS (1% w/v) and protease (Sigma, 1 mg/ml) and incubation continued for 45min at 37^{o}C. An equal volume of phenol and chloroform were added to the suspension and the mixture was shaken and centrifuged (8 000g, 10min). The top aqueous layer was decanted and denatured at 100^{o}C for 10min then transferred to ice to produce single stranded fragments. DNA fragments between 5 and 12 nucleotides long were collected by separating the mixture on a Whatman DE 52 cellulose column (20cm x 1cm). The column was equilibrated with equilibration buffer before the DNA fragment mixture was loaded and washed through with the equilibration buffer until all the OD_{258} absorbing material was washed free. This removed all the fragments of up to 5 nucleotides in length. Fragments between 5 and 12 nucleotides in length were eluted by washing the column with elution buffer until all the OD_{258} absorbing material

was washed free. This fraction was ethanol precipitated by the addition of 0.2M NaCl, 2 volumes of ethanol and the mixture was held at -70^{0}C overnight. The precipitated fragments were resuspended in deionised water at a concentration of 50 ug/ml.

2.11 Preparation of Biotin-labelled Probe DNA (modified from Whitfeld, 1982)

2.11.1 Materials

HaeIII Buffer (10x stock) contained 100mM Tris, 80mM $MgCl_2$ and 14mM mercaptoethanol. The pH was adjusted to 7.5 with HCl.

Sodium Chloride stock solution was 1M NaCl.

EDTA stock solution was 0.25M EDTA.

Sodium Acetate stock solution was 3M sodium acetate.

Ethanol was 95% ethanol.

2.11.2 Method

The DNA used to make the probe was digested in a 25ul reaction mix which contained: DNA, 2ug; 10x HAEIII buffer, 2.5ul; 1M NaCl, 2.5ul; HAEIII (New England Biolabs), 2ul. The reaction mixture was incubated at 37^{o}C for 30min. Random primers (4ul) and deionised water (10ul) were added to the digest and it was heated to 100^{o}C for 2-3min and immediately cooled to 0^{o}C in ice. The following were then added to the digest; 10x HAEIII buffer, 2ul; 2-deoxyguanosine-5'-triphosphate (dGTP) (Sigma 20mM), 1ul; 2-deoxyadenosine-5'-triphosphate (dATP) (Sigma 20mM), 1ul; 2-deoxycytosine-5'-triphosphate (dCTP) (Sigma 20mM), 1ul; 5-[N-(N-biotinyl-E-aminocaproyl)-3-aminoallyl]-deoxyuridine-triphosphate (bio-11-dUTP) (ENZO Biochem 0.3mM), 4ul; DNA polymerase 1 (Klenow fragment) (BRL), 2ul. The mixture was incubated at 37^{o}C for 30min, then the reaction was stopped by the addition of 3ul of 0.25M EDTA. The probe DNA was precipitated with ethanol by the addition of 5ul of 3M NaAc and 500ul of ethanol, mixed by inversion and held

at -16°C overnight. It was centrifuged for 15min at 9 000g, and the supernatant was discarded. The pellet was dried in a vacuum dessicator for 15min, resuspended in 100ul of sterile deionised water and stored at 4°C.

2.12 Preparation of a ^{32}P-Labeled DNA Probe by the Random Priming Method (Whitfield, 1982)

The procedure for the preparation of a ^{32}P labeled probe was the same as for a biotin-labelled probe except that 1ul of 2-deoxythymidine-5'-triphosphate (dTTP) (Sigma 20mM) was substituted for 4ul of bio-11-dUTP and 3ul of deoxycytosine-5'-(a-^{32}P)-triphosphate (dCT^{32}P) (New England Nuclear, 30Ci) was substituted for 1ul of 20mM dCTP. The ^{32}P probe was not ethanol precipitated, but it was treated in a mini-spin column.

2.13 Separation of ^{32}P-Labelled DNA from Unbound Nucleotides using a "Mini-spin" Column

2.13.1 Materials

Tris EDTA Sodium Chloride buffer (TES buffer) contained 10mM Tris, 1mM EDTA and 0.1M NaCl. The pH was adjusted to 8.0 with HCl.

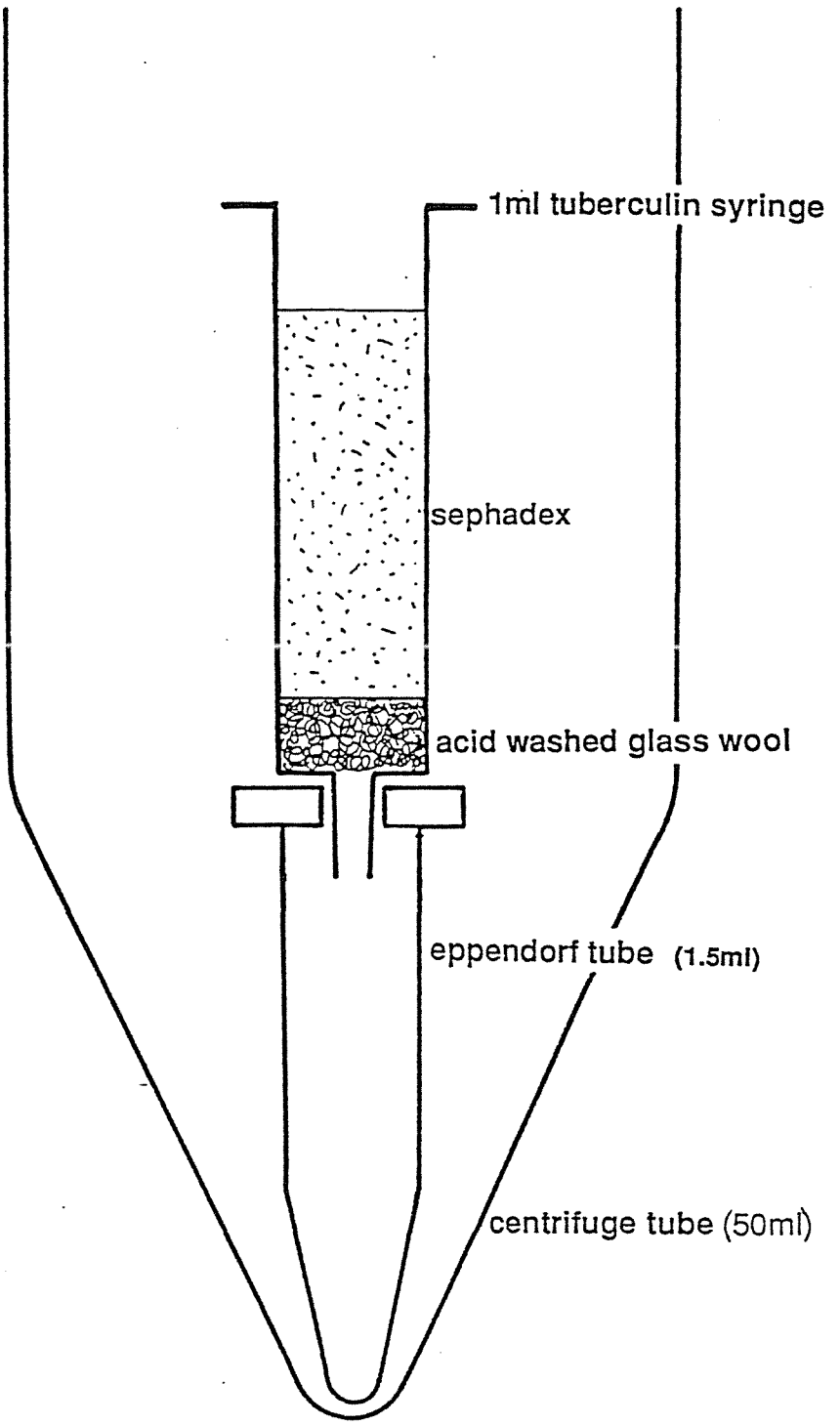
Acid washed glass wool was prepared by soaking the glass wool in 30% Nitric acid then rinsing it in deionised water until the pH was neutralised.

Hydrochloric Acid was 2M HCl.

2.13.2 Method

The mini-spin column was made by taking a 1ml disposable syringe and packing the outlet to about 0.05ml with acid washed glass wool. Sephadex G50-80 (Sigma), which has been preswollen in TES buffer according to the manufacturers instructions, was pipetted into the syringe until it was full. The syringe outlet was inserted into the cap of an Eppendorf tube, placed in a 50ml conical centrifuge tube and centrifuged for 2min at 12 000g in a bench centrifuge fitted with a swinging rotor. The liquid

Figure 4: A sephadex G50-80 "mini-spin" column used for probe purification (see section 2.2.13 for details).


1ml tuberculin syringe
sephadex
acid washed glass wool
eppendorf tube (1.5ml)
centrifuge tube (50ml)

which collected in the Eppendorf tube was discarded and more Sephadex suspension was added until the volume of Sephadex after cetrifugation was at least 0.8ml (Figure 4). Immediately prior to use 100ul of TES buffer was passed through the column and discarded. The DNA labelling reaction mix was added to the top of the column along with 100ul of TES buffer and centrifuged for 2min at 12 000g. The column retained most of the unincorporated ^{32}P while the labelled DNA collected in the tube. The column was discarded and the probe DNA checked for incorporated counts by placing a 2ul sample near the base of a polyethyleneimine (PEI) strip. The strip was placed vertically in a beaker containing a small volume of 2M HCl. When the liquid had nearly reached the top of the strip it was removed and cut in half. Unincorporated ^{32}P travelled to the top of the strip while the labelled DNA remained at the bottom. Each half was placed in a scintillation vial containing 10ml of water and the radioactivity associated with it was measured in a scintillation counter (Beckman LS7000). The total number of counts incorporated in the probe DNA was then calculated.

2.14 Hybridisation of ^{32}P-Labelled and Biotin-Labelled DNA Probes to Nitrocellulose-bound DNA

2.14.1 Materials

Hybridisation Buffer (modified Denharts buffer, Jarvis et al, 1983) contained per litre: 1M Hepes buffer (Sigma) pH 7, 50ml; 20xSSC, 150ml; 3 mg/ml herringsperm DNA, 6ml; 10 mg/ml *E coli* transfer RNA (Sigma), 2ml; 20% SLS, 5ml; Ficoll (Sigma type 70), 2g; Bovine Serum Albumin (Sigma), 2g; Polyvinyl pyrrolidone (PVP-10 Sigma), 2g.

20X SSC was 3M NaCl and 0.3M sodium citrate. The pH was adjusted to 7.0 with NaOH.

2.14.2 Method

Prehybridisation. The nitrocellulose membrane was placed in a plastic bag with approximately 15ml of hybridisation buffer, the air bubbles were removed and the bag heat-sealed. The bag was incubated at 60^{o}C for 2hr.

Hybridisation. The probe was placed in an Eppendorf tube with a perforated cap, heated to 100°C for 1min and transferred immediately to ice. A corner was removed from the bag containing the prehybridised filter and about half the hybridisation buffer was poured out. The labelled probe DNA was added and the bag sealed. The contents of the bag were mixed to ensure an even distribution of the probe and the bag was incubated at 60°C overnight.

Washing Procedure to Remove Unbound Probe. After hybridisation, the nitrocellulose membrane was removed from the plastic bag, washed twice in 2xSSC for 15min at room temperature and twice in 0.1xSSC for 15min once at room temperature and once at 50°C.

2.15 Detection of Hybridisation on DNA Blots Hybridised with Biotin-labelled Probes (according to manufacturers instructions, ENZO Biochem BIO-NOTE 315-02-8507)

2.15.1 Materials

Phosphate Buffered Saline (PBS) contained 1.3M NaCl, 70mM Na_2HPO_4 and 30mM NaH_2PO_4.

Blocking Buffer contained 1x PBS, 5mM EDTA (pH7.5), 2% BSA (filter sterilised) and 0.05% Triton X-100.

Complex Dilution Buffer contained 1x PBS, 5mM EDTA (pH7.5), 0.5% Triton X-100 and 0.1% BSA (filter sterilised).

Washing Buffer contained 10mM K_2HPO_4/KH_2PO_4 pH6.5, 0.5M NaCl, 1mM EDTA (pH7.5), 0.5% Triton X-100 and 2% BSA (filter sterilised).

Predetection Buffer contained 100mM Tris pH8.8, 100mM NaCl and 5mM MgCl.

Substrate Stock Solution (BCIP stock solution) contained 50mg of 5Bromo-4Chloro-3Indolyl phospate (BCIP) (Sigma) dissolved in 1ml anhydrous dimethyl formamide. The solution was stored at 4°C in the dark.

Dye Stock Solution (NBT stock solution) contained 75mg of Nitro Blue Tetrazolium (NBT) (Sigma) suspended in 0.7ml of anhydrous dimethyl formamide. Deionised water (0.3ml) was added to dissolve the NBT. The solution was stored at 4^{o}C in the dark.

Termination Buffer (Leary, 1983) contained 10mM Tris and 1mM EDTA. The pH was adjusted to 7.5 with HCl.

2.15.2 Method

After the nitrocellulose membrane was washed it was submerged in blocking buffer for 30min at room temperature and then placed in a plastic bag. The excess blocking buffer was removed. 1ml of complex dilution buffer to which 6ul of the streptavidin-alkaline phosphatase conjugate (Detek 1-alk, ENZO Biochem or SA-AP-conjugate, BRL) had been added was put into the bag. Air was excluded and the sealed bag left at room temperature for 1hr. The membrane was removed from the bag and shaken in five changes of washing buffer, for 5min per change, and then in two changes of predetection buffer for 3min per change. The membrane was submerged in a freshly prepared reaction mix containing 25ml of predetection buffer, 12.5ul of NBT stock solution and 75ul BCIP stock solution. To stop the colour reaction the membrane was washed in termination buffer and placed on a piece of filter paper to dry. When developed, slot blots were read on a scanning densitometer (LKB UltroScan XL) with the following settings: "line" for type of beam, "yes" for smoothing and "2" for peak-width. An example of the resulting print-out is given in figure 5.

2.16 Detection of Hybridisation on DNA blots hybridised with a ^{32}P-Labelled Probe

The washed membrane was placed on a piece of Whatman 3MM filter paper to dry, attached to a piece of filter paper cut to fit an 18cm x 24cm cassette, covered with Gladwrap and placed in a cassette next to a piece of X-ray film (Agfa-Gevaert Curix RP2) between two intensifying screens. The cassette was left at -70^{o}C for 48hr for the initial exposure. The film was developed in a Kodak automatic film processor.

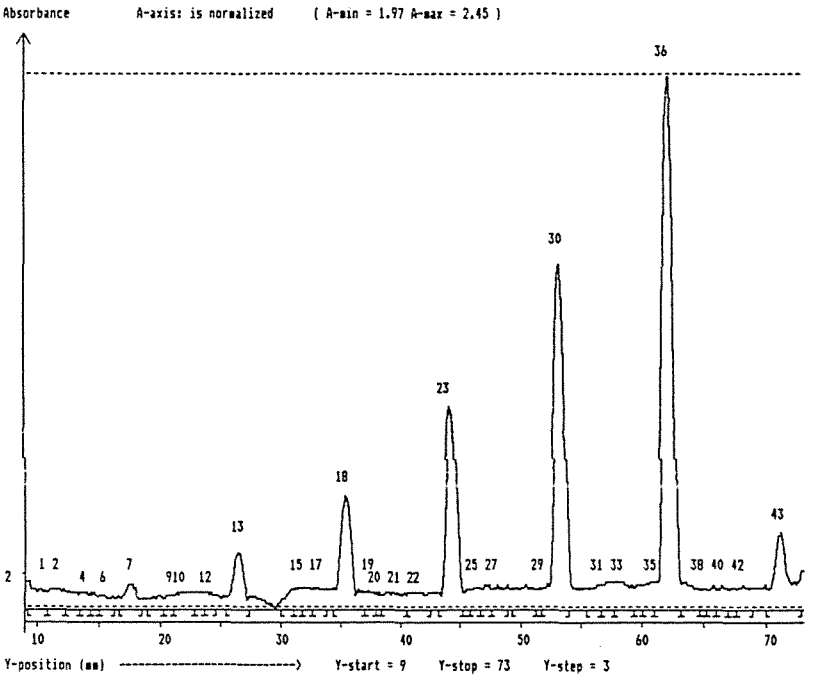
Figure 5: An example of a print out from the LKB scanning densitometer of a slot-blot containing genomic *Rhizobium leguminosarum* biovar *trifolii* strain ICMP2668 DNA hybridised at 60°C for 20hr with a homologous biotin-labelled probe and visualised with a steptavidin/alkaline phosphatase conjugate under standard development conditions.

peak	DNA	ug
43	*E.coli*	1.000
36	ICMP2668	1.000
30	ICMP2668	0.500
23	ICMP2668	0.250
18	ICMP2668	0.125
13	ICMP2668	0.063
07	ICMP2668	0.031

LKB UltroScan XL

Run by Operator: £ 17 Run ID £ = 2319 Tuesday Dec-22-1987 15:07

Track = 4 of 6 X-position = 72.2 X-width = 6
Type of beam = line Smoothing: YES Peak-width = 2
Base line: at 1.97 AU is average of the 16 lowest data points (type = 1)



PEAK £	POSITION mm	HEIGHT AU	AREA AU*mm	REL. AREA %	PEAK £	POSITION mm	HEIGHT AU	AREA AU*mm	REL. AREA %
1	10.2	0.01	0.03	1.3	23	44.2	0.18	0.18	9.7
2	11.3	0.01	0.02	1.2	24	45.8	0.01	0.01	0.5
3	13.0	0.01	0.02	0.9	25	46.4	0.02	0.02	0.8
4	13.7	0.01	0.01	0.6	26	47.1	0.02	0.02	1.0
5	14.4	0.01	0.01	0.4	27	48.0	0.02	0.02	1.3
6	15.3	0.01	0.01	0.6	28	50.3	0.02	0.03	1.7
7	17.6	0.02	0.03	1.3	29	51.6	0.01	0.01	0.5
8	20.0	0.01	0.01	0.6	30	53.1	0.31	0.31	16.4
9	20.9	0.01	0.01	0.5	31	56.4	0.02	0.02	1.3
10	21.9	0.01	0.02	1.1	32	57.3	0.02	0.03	1.4
11	23.7	0.01	0.01	0.6	33	58.1	0.02	0.03	1.8
12	23.7	0.01	0.01	0.6	34	59.8	0.02	0.02	0.9
13	26.4	0.05	0.05	2.7	35	60.8	0.02	0.02	1.2
14	31.0	0.01	0.01	0.7	36	62.1	0.48	0.48	25.2
15	31.6	0.02	0.01	0.7	37	63.3	0.02	0.03	1.5
16	32.1	0.02	0.02	0.8	38	64.8	0.02	0.01	0.6
17	32.9	0.02	0.02	1.1	39	65.6	0.02	0.01	0.7
18	35.3	0.10	0.12	6.1	40	66.3	0.02	0.02	0.9
19	37.2	0.01	0.01	0.7	41	67.2	0.02	0.01	0.7
20	38.1	0.01	0.01	0.3	42	68.0	0.02	0.02	1.3
21	39.5	0.01	0.02	1.3	43	71.0	0.06	0.10	5.2
22	41.6	0.01	0.02	1.3					

2.17 Washing Bioprobed Blots for Probing with a Different Bioprobe

2.17.1 Materials

Denaturing Solution was 20mM NaOH.

Neutralising Solution contained 0.5M Tris and 2M NaCl. The pH was adjusted to 7.4 with HCl.

20xSSC contained 3M NaCl and 0.3M sodium citrate. The pH was adjusted to 7.0 with NaOH.

2.17.2 Method

A bioprobed blot that had undergone detection was washed by shaking in denaturing solution, neutralising solution and 2xSSC for 20min, 15min and 15min respectively. The blot was then placed on a piece of filter paper to dry.

2.18 The Preparation of Blots from Colonies or Nodules (Cooper, 1987)

2.18.1 Materials

Proteinase stock solution contained (per ml): SLS, 10mg; Proteinase K (Boehringer), 200ug.

Denaturing Solution contained 1M NaOH and 3M NaCl.

Neutralising Buffer contained 0.5M Tris and 1.5M NaCl. The pH was adjusted to 7.4 with HCL.

Washing Buffer contained 50mM Tris, 1M NaCl, 1mM EDTA and 0.1% w/v SLS.

20xSSC contained 3M NaCl and 0.3M sodium citrate. The pH was adjusted to 7.0 with NaOH.

2.18.2 Method

Nodules were sterilised (section 2.2.3), crushed in 40ul of sterile water and spotted onto nitrocellulose. Colonies were either transferred directly to nitrocellulose or mixed in 40ul of sterile water and spotted onto nitrocellulose. If a higher concentration of cells was required sterile nitrocellulose (121^{o}C, 10min) was inoculated and placed on a TY agar plate for incubation at 27^{o}C for 24-48hr.The prepared nitrocellulose was placed on filter paper (15cm x 20cm) saturated with proteinase solution for 1hr at 37^{o}C; transferred to paper saturated with denaturing solution for 5min at room temperature and then to paper saturated with neutralising buffer for 5min at room temperature. The membrane was allowed to dry between each treatment. The nitrocellulose was baked for 2hr at 80^{o}C in a vacuum oven. Before prehybridisation the nitrocellulose was rewetted from below with 6xSSC for 10min and then washed in washing buffer for 1hr at 57^{o}C.

2.19 Growth of a Gene Library from *Bradyrhizobium* sp. *(Lotus)* strain cc814S

A gene library of 20 kilo-base inserts of cc814S in pLAFR1 was obtained from Prof. D. B. Scott. A dilution plate count was made on LB^{TC} agar (section 2.2.1) plates and incubated at 37^{o}C for 24hr to determine the viable cell concentration. The probability of getting a clone was calculated with the formula

$$N = \frac{\ln(1 - P)}{\ln[1 - (I/G)]}$$

where N = the number of colonies required
P = the probability (percentage)
I = size of the cloned inserts (Kb)
G = genome size (Kb)

Where P = 95%, I = 20Kb and G = 6 000Kb the number of colonies required is 900. The appropriate dilution to give approximately 300 colonies per plate was calculated and plated onto LB^{TC} agar in three large (13.5cm diameter) glass petri dishes. They were incubated at 37^{o}C for 24hr.

2.20 Colony Lifts from a Gene Library

2.20.1 Materials

Sodium Hydroxide stock solution was 0.5M NaOH.

Neutralising buffer contained 1M Tris and 1.5M NaCl. The pH was adjusted to 7.4 with HCl.

20xSSC contained 3M NaCl and 0.3M sodium citrate. The pH was adjusted to 7.0 with NaOH.

2.20.2 Method

Nitrocellulose membranes cut to cover the area containing the colonies of the gene library were wetted with deionised water, placed between sheets of filter paper, autoclaved for 10min at 121^{o}C and left to dry. Each nitrocellulose membrane was placed onto the surface of a separate plate then peeled off. The plates were reincubated for a further 24hr at 37^{o}C then stored at 4^{o}C. The nitrocellulose membranes were placed on a piece of filter paper soaked in 0.5M NaOH for 7min, then on filter paper soaked in neutralising buffer until a pH strip indicated that they were neutralised. The membranes were washed in 4 changes of 2xSSC, dried on a piece of filter paper and baked for 2hr at 80^{o}C in a vacuum oven.

2.21 Hybridisation of a ^{32}P Probe to Dissolved Unlabelled DNA

2.21.1 Materials

Phosphate Buffer (1.4M) stock solution contained (g/l): Na_2HPO_4, 99.4; NaH_2PO_4, 109.2. The pH was adjusted to 6.8 with HCl.

2.21.2 Method

3ug of DNA labelled with ^{32}P (section 2.2.12) suspended in 100ul was added to 150ug of cold DNA disolved in deionised water and the mix was made up to 1ml

with Phosphate Buffer to give a final concentration of 0.1M Phosphate Buffer. The mix was floated in boiling water for 7min, transferred to ice for 5min and incubated at 65°C for 27hr.

2.22 Separation of Single-stranded DNA from Double-stranded DNA with Hydroxylapatite

2.22.1 Materials

Phosphate Buffer (1.4M) stock solution contained (g/l): Na_2HPO_4, 99.4; NaH_2PO_4, 109.2. The pH was adjusted to 6.8 with HCl.

Sodium Lauryl Sulphate (SLS) stock solution was 0.4% SLS.

2.22.2 Method

Hydroxylapatite (Bio-Rad) (0.7g) was washed twice, with 8ml of 0.1M Phosphate Buffer containing 0.4% SLS, by mixing it with an overhead stirrer (Multispeed, Anderson and Co., Central Ave, East Mulsey, Surrey, England), centrifuging for 1min and discarding the supernatant. The hybridisation mix (1ml) was added to the hydroxylapatite, mixed thoroughly with the stirrer and placed in a circulating waterbath (Haake Type F, Haake Circulators, Berlin, Germany) at 65°C. When the temperature in the tube reached 65°C it was removed to a heated centrifuge at 65°C and centrifuged at 1 000g for 1min and the supernatant was immediately poured into a scintillation vial. This process was repeated three more times using 2ml of 0.1M Phosphate Buffer in place of the hybridisation mix. Then 8ml of 0.4M Phosphate Buffer was added to the hydroxylapatite and it was left at room temperature for 4min, centrifuged for 1min and the supernatant poured into a scintillation vial. This process was repeated three times. The eight samples collected were counted on a scintillation counter. The first four samples contained single-stranded DNA and the last four samples contained double-stranded DNA.

2.23 The Rapid Boil Method for the Isolation of Cosmid DNA (Holmes and Quigley, 1981)

2.23.1 Materials

Sucrose Triton X EDTA Tris Buffer (STET buffer) contained (per 100ml): sucrose, 8g; triton X-100, 5ml; 250mM EDTA pH8, 20ml; 1M Tris, 5ml.

Lysozyme Solution contained lysozyme at a concentration of 10mg/ml in 10mM Tris pH7.6.

Ethanol was 95% ethanol.

2.23.2 Method

Luria broth containing tetracycline (section 2.2.1) (2.5ml) was inoculated with a single colony and grown overnight on a shaker at 37°C. Part of this culture (1.4ml) was pelleted and the supernatant discarded. The cells were resuspended in 350ul of STET Buffer to which 25ul of freshly prepared lysozyme solution was added. The solution was boiled for 40sec and immediately centrifuged (9 000g, 10min). The resulting pellet was removed with a sterile toothpick and discarded. An equal volume of isopropanol was added to the supernatant to precipitate the plasmid DNA. This solution was mixed by inversion and left at -16°C for 10min, then centrifuged (9 000g, 5min) and the supernatant discarded. The pellet was washed with ethanol, dried in a vacuum desiccator for 5min and resuspended in 50ul of sterile deionised water. If it was not used immediately it was stored at 4°C.

2.24 Isolation of Cosmid DNA by Potassium Acetate Precipitation (modified Ish-Horowicz and Burke, 1981)

2.24.1 Materials

Solution I contained (per 500ml): glucose 4.95g; 1M Tris pH 8, 12.5ml; 250mM EDTA, 20ml. This solution was autoclaved for 15min at 121°C.

Solution II was 1% SLS and 0.2M NaOH.

Solution III contained (per 100ml): Potassium Acetate, 29.44g; Glacial Acetic Acid, 11.5ml.

Lysozyme Solution contained lysozyme at a concentration of 50mg/ml in 10mM Tris pH7.6.

Ethanol was 95% ethanol.

Tris EDTA Buffer (TE Buffer) contained 50mM Tris and 20mM EDTA. The pH was adjusted to 8.0 with HCl.

2.24.2 Method

Figures in <> refer to a mini preparation, figures in [] refer to a bulk preparation.

Luria broth containing tetracycline (section 2.2.1) <10ml> [1000ml] was inoculated with an *E. coli* strain containing the required plasmid and incubated on a shaker at 37°C overnight. The culture was centrifuged at <10min, in a microfuge> [10min, 8 000g] and the supernatant discarded. The cells were resuspended in Solution I <100ul> [30ml] containing fresh lysozyme solution <10ul> [3ml] and left at room temperature for 5min. Solution II <200ul> [60ml] was added and the mixture was held on ice for 5min. Solution III <150ul> [45ml] was added, the mixture was left on ice for 5min, centrifuged for <5min, in a microcentrifuge> [10min, 7 500g] and the supernatant decanted into a clean centrifuge tube. Isopropanol <240ul> [82.5ml] was added to the supernatant to precipitate the plasmid DNA and it was left at room temperature for 5min then centrifuged <10min, in a microcentrifuge> [20min, 8 000g] and the supernatant discarded. The pellet was washed twice with ethanol, dried in a vacuum desicator for 15min and resuspended in <30ul of sterile deionised water> [10ml of TE buffer]. If not used immediately the plasmid preparation was stored at 4°C.

2.25 Transfer of DNA to Nitrocellulose by Capillary Blotting (Southern, 1975)

2.25.1 Materials

Hydrochloric Acid was 0.25M HCl.

Denaturing Solution contained 0.5M NaCl and 0.5M NaOH.

Neutralising Buffer contained 0.5M Tris and 2M NaCl. The pH was adjusted to 7.2 with HCl.

20xSSC contained 3M NaCl and 0.3M sodium citrate. The pH was adjusted to 7.0 with NaOH.

2.25.2 Method

The gel was soaked in HCl for 15min, washed with deionised water, soaked in denaturing solution for 30min, washed with deionised water and soaked in neutralising buffer for 30min. The blotting apparatus was prepared (Figure 6) by placing 4 sheets of filter paper (20cm x 15cm) in a Pyrex baking dish and soaking these in 20xSSC. A piece of Gladwrap^{TM} was placed over the filter paper with the edges wrapping over the edges of the dish. A hole that was 5mm smaller than the gel on all sides was cut in the Gladwrap^{TM} with a scalpel blade. The gel was placed over this hole and a piece of prewetted nitrocellulose cut slightly larger than the gel was placed on top of the gel making sure there were no air bubbles between the nitrocellulose and the gel. A piece of filter paper, cut slightly smaller than the nitrocellulose, was prewetted with 20xSSC and placed on the nitrocellulose so that no air bubbles where trapped between the membrane and the paper. Two more pieces of filter paper were placed on top of the prewetted sheet. A stack of paper toweling was placed on top of this with a glass plate and a 500g weight on top to provide even pressure. The filter paper under the Gladwrap^{TM} was flooded with 20xSSC and the apparatus left at room temperature overnight. Then the nitrocellulose was removed, washed in 2xSSC for 15sec, dried and baked for 2hr at 80^{o}C in a vacuum oven.

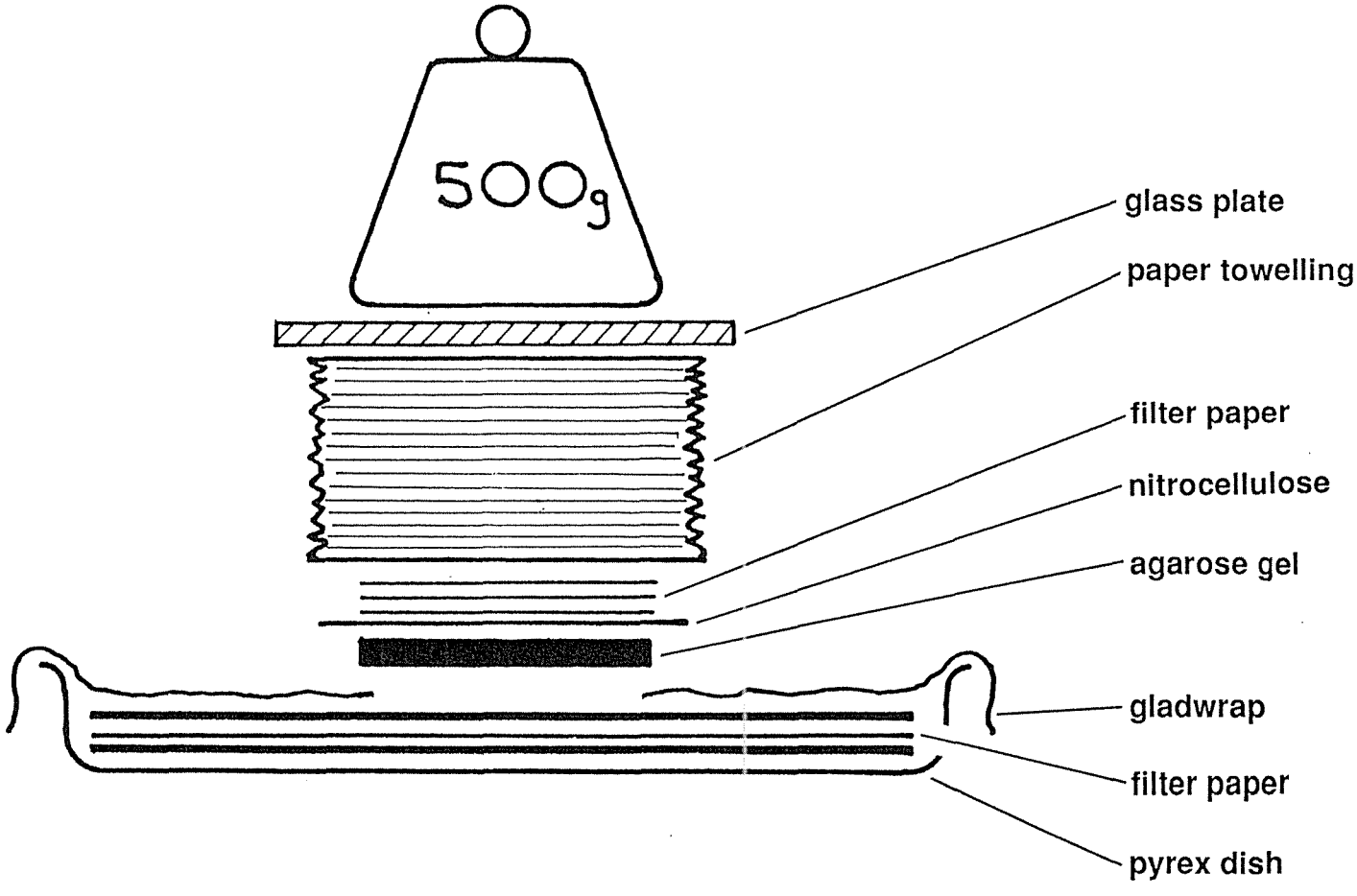
2.26 **Purification of Plasmid DNA on a Cesium Chloride Gradient** (Davis, 1980)

2.26.1 Materials

Ethidium Bromide stock solution contained ethidium bromide at a concentration of 10 mg/ml.

TE Buffer contained 50mM Tris and 20mM EDTA. The pH was adjusted to 8 with HCl.

Figure 6: The apparatus used for DNA transfer from agarose gels to nitrocellulose membranes by Southern blotting (see section 2.2.25 for details)


500g
glass plate
paper towelling
filter paper
nitrocellulose
agarose gel
gladwrap
filter paper
pyrex dish

2.26.2 Method

Cesium Chloride (10.25g) was added to a bulk plasmid preparation (section 2.2.24) and dissolved at room temperature. Ethidium bromide (0.65ml) was added and a precipitate formed (from here on the sample was kept in subdued light to avoid nicking the DNA). The sample was centrifuged (10 000g, 20min, 15-20oC), the refractive index adjusted to 1.3885 and the supernatant decanted into Sorvall ultracentrifuge tubes for the vertical rotor TV865. the sample was centrifuged at 55 000g for 5hr on a Beckman Model L5-75 Ultracentrifuge. The plasmid band was visualised with long wave UV light and removed by inserting a 1ml "Tuberculin" syringe with a 22 gauge needle just below the band and applying suction. The ethidium bromide was extracted twice with isopropanol saturated with CsCl and TE buffer (from here it was possible to work in normal light). The sample was dialysed against three 500ml changes of TE buffer over a 28hr period at 4^{o}C. The DNA concentration was calculated (section 2.2.5) and the sample precipitated with ethanol (section 2.2.6) and resuspended in sterile deionised water to give a final concentration of 0.5 mg/ml.

2.27 Digestion of DNA with an Endonuclease

A 25ul reaction mixture contained: DNA, 2-5 ug; endonuclease buffer (as specified by the manufacturer of the endonuclease), 2.5ul; endonuclease, 2ul. The reaction mixture was incubated for 1hr at 37^{o}C. If the digestion had not proceeded to completion at this stage then another 2ul of endonuclease were added and the incubation continued for a further hour at 37^{o}C. The digestion was stored at 4^{o}C. If a larger digestion was required the reaction mixture could be multiplyed accordingly.

2.28 Removal of the 5' Phosphate from Linear DNA (CAP Treatment)

2.28.1 Materials

Sodium Lauryl Sulphate (SLS) stock solution was 25% SLS.

Proteinase K stock solution contained proteinase K (Boehringer) at a concentration of 50 mg/ml.

Phenol/Chloroform contained equal volumes of redistilled (section 2.2.41) phenol and chloroform.

Sodium Acetate stock solution was 3M NaAc.

Ethanol was 95% ethanol.

2.28.2 Method

To prevent the religation of the digested vector to itself the 5' phosphate was removed by CAP treatment. To the digested vector DNA (section 2.2.27) 1ul of Calf Alkaline Phosphatase (CAP) (Boehringer) was added and the mixture was incubated at 37°C for 30min. A further 1ul of CAP was added and the incubation was continued at 37°C for 30min. The mixture was made 1% with respect to SLS and 0.5 mg/ml with respect to proteinase K and incubated at 37°C for 30min to digest the CAP enzyme. The enzyme protien was extracted with phenol/chloroform by the addition of an equal volume of phenol/chloroform, mixed by inversion and centrifuging (in a microfuge, 5min). The upper phase was removed to a new tube which was then extracted with chloroform in a similar manner. The mixture was then ethanol precipitated (section 2.2.6) and resuspended in deionised water at the desired concentration.

2.29 Ligation of Vector DNA with Insert DNA (Dugaiczyk, 1975)

2.29.1 Materials

10X Ligation Buffer contained 60mM Tris, 100mM $MgCl_2$ and 100mM mercaptoethanol. The pH was adjusted to 7.6 with HCl.

2.29.2 Method

The ligation mixture contained: vector DNA (pGem2 digested with BAMH1 and CAP treated), 3ul; insert DNA (digested), 1ul; 10X ligation buffer, 1ul; ligase (BDH), 1ul. This was incubated at 14°C overnight and half the mixture was separated by electrophoresis on an agarose gel (section 2.2.7) to determine whether ligation had occured.

2.30 Electroporation of a Vector into *E.coli* strain HB101 Cells

2.30.1 Materials

Glycerol stock solution was 10% glycerol.

SOC Buffer was 2% tryptone, 0.5% Yeast extract, 1mM NaCl, 2.5mM KCl, 10mM MgCl, 10mM $MgSO_4$ and 20mM glucose.

2.30.2 Method

***E.coli* HB101 cells** were prepared by inoculating 1 litre of LB (section 2.2.1) with a 10ml broth culture of strain HB101 and incubating on a shaker at 37°C for 3hr. The cells were then chilled on ice and centrifuged at 4 000rpm at 4°C for 15min, resuspended in 1 litre of cold deionised water, recentrifuged, resuspended in 0.5 litre of cold deionised water, recentrifuged, resuspended in 20ml of glycerol solution, recentrifuged and resuspended in 1-4ml of glycerol solution and stored at -16°C.

The ligation mix (3ul) was added to 40ul of *E.coli* HB101 cells and placed in a Potter-type cuvette with a 0.2cm gap between the electrodes. The cuvette was placed in the safety chamber of a Gene Pulser^{TM} apparatus (Bio-Rad Laborataries, Richmond, CA) and a pulse of 2.48 volts applied. Following the pulse, the cells were immediately removed from the cuvette to a 1.5ml polypropylene tube and SOC buffer (1ml) was added. The cells were incubated on a shaker at 37°C for 1hr, then spread plated onto LB^{AMP} agar (section 2.2.1) plates and incubated at 37°C for 24hr.

2.31 Growth of a Plasmid Library of Isolate JG210

A plasmid library of isolate JG210 (400-600 base pair inserts) in pGEM2 was obtained by electroporating the ligation mix into *E.coli* HB101 cells. The resulting colonies were picked and patched onto LB^{AMP} agar in large glass (13.5cm diameter) plates. A colony lift (section 2.2.20) was made of each plate which was hybridised with a ^{32}P-labeled insert DNA probe, the colonies that reacted were patched onto further plates. The number of colonies required to give a 95% probability of obtaining a specific fragment was calculated with the formula

$$N = \frac{\ln(1 - P)}{\ln[1 - (I/G)]}$$

where N = the number of colonies required

P = the probability (percentage)

I = size of the cloned inserts (Kb)

G = genome size (Kb)

When P = 95, I = 0.5 and G = 45 268 colonies required to represent the isolate JG210 plasmid.

RESULTS

3.1 IDENTIFICATION OF *RHIZOBIUM* AND *BRADYRHIZOBIUM* SPECIES BY HYBRIDISATION OF A BIOTIN-LABELLED GENOMIC DNA PROBE TO GENOMIC DNA BOUND TO NITROCELLULOSE

Development of the non-radioactive biotin-streptavidin/alkaline phosphatase nucleic acid detection system as described by ENZO Biochem and Bethesda Research Laboratory for the identification of *Rhizobium* and *Bradyrhizobium* species required an analysable result. To achieve this it was necessary to have control over the colour development and to use a system in which the results could be measured. The Bio-Blot^{R} SF Blotting Apparatus (Bio-Rad Laboratories, Richmond, California) produced blots that could be read on an LKB UltroScan XL scanning densitometer which converted the intensity of the colour into a numerical figure. Variables that affected the colour intensity produced were investigated to ascertain which conditions gave the best result for this application. Alterations were made to the method to decrease the cost and the hybridisation time. The method was compared with a conventional ^{32}P-labelling system and applied to colony and nodule extract hybridisations.

3.1.1 Control of Colour Development

When a blot is removed from the detection mix and placed on a piece of filter paper to dry the enzyme continues to develop colour until the blot is completely dry. This may result in over developement of the blot in which case the negative control will show a reaction and the net result (sample minus control) decreases. To determine the best method for stopping the development of colour at the desired time three identical dot-blots were made using DNA from *E. coli* (negative control), *Rhizobium* strains ICMP2668, Gal 14, U45 and *Bradyrhizobium japonicum* strain NZP5536 at five two fold dilutions ranging in concentration from 0.0625ug to 1ug per spot. All three blots were hybridised with a biotin-labelled ICMP2668 DNA probe at 60^{0}C for 20hr and developed for an equal period of time. One blot was placed on a piece of filter paper to dry at room temperature, one blot was dried in a vacuum oven at 80^{o}C and one was rinsed in 10:1 TE buffer pH 7.5 twice and placed on a piece of filter paper to dry at room temperature. The results are shown in Table IV.

TABLE IV : The Efficencies of Different Methods of Terminating the Colour Reaction of the Streptavidin/Alkaline Phosphatase Conjugate used to Visualise Biotin-labelled Genomic DNA Probes Hybridised at 60°C for 20hr with Homologous DNA Dot-blots

TREATMENT	DNA CONCENTRATION (ug/spot)	ORIGIN OF DNA				
		E. coli	NZP2668	Gal 14	NZP5536	U45
A. Blot Air Dried	1.0000	+	++	+	+	+
at Room	0.2500	+	++	+	+	+
Temperature	0.1250	-	++	+	+	-
	0.0625	-	+	+	-	-
B. Blot Oven Dried	1.0000	+	++	++	+	+
80°C in vacuo	0.2500	+	++	+	+	+
	0.1250	-	++	-	-	-
	0.0625	-	+	-	-	-
C. Blot Washed in	1.0000	-	++	+	-	-
10:1 TE Buffer	0.2500	-	+	-	-	-
pH 7.5 Air Dried	0.1250	-	-	-	-	-
at Room	0.0625	-	-	-	-	-
Temperature						

- = the absense of colour; + = some colour; ++ = intense colour.

The air dried and the oven dried blots both continued colour development after being removed from the reaction mixture and this resulted in a non-specific reaction occuring as the blot became over-developed and colour was produced in the negative control. The blot washed in TE buffer stopped colour deelopment immediately after removal from the reaction mix.

3.1.2 The Effect of Ethanol Washing on Dot-Blot Quality

The washing of dot-blots with 90% ethanol under vacuum to dissolve and remove material that may produce a non-specific reaction (section 2.2.82) resulted in excessive permanent wrinkling of the nitrocellulose membrane. This made the results harder to interpret and photograph than if the membrane was flat. To determine whether it was possible to omit this step without loss of specificity two identical blots were prepared using DNA from *E. coli* (negative control), and *Rhizobium* strains ICMP2668, NZP5232, U45, 505/1 and NZP5224 at five two fold dilutions ranging in concentration from 0.0625ug to 1ug per spot. One blot was washed with ethanol the other was not. Both blots were hybridised with a biotin-labelled ICMP2668 DNA probe at 60^0C for 20hr and developed for an equal period of time. There was no visible difference in the intensity of the color produced, the detection limit or the background between the two blots.

3.1.3 Removal of Colour After Detection of a Biotin-Labelled Probe

To determine if the blue precipitate of Diformazan produced from the reaction of 5Bromo-4Chloro-3Indolyl-phosphate and nitro blue tetrazolium catalysed by alkaline phosphatase (McGrady, 1970) could be removed from a dot blot, so that it could be hybridised with a different probe, a developed dot blot that had been allowed to dry was washed (section 2.2.16). This gave no discernible decrease in colour intensity. To determine if this was due to the fact that the blot had been allowed to dry before washing a dot blot was washed immediately after development. Again there no discernible difference in colour was produced.

3.1.4 Substitution of Skimmed Milk Powder for Bovine Serum Albumin as a Blocking Agent.

Bovine Serum Albumin (BSA) is a component of three of the buffers used in the detection of hybridisation with a biotin-labelled probe (the blocking buffer, complex detection buffer and washing buffer). To decrease the cost of detecting a biotin-labelled probe a substitute for BSA was sought. Milk powder has been used as a substitute for BSA in other applications. To determine if it would be an effective substitute for BSA in the detection of hybridisation five identical dot blots were prepared using DNA from *E. coli* (negative control), *Rhizobium* strains ICMP2668, U45, 505/1 and NZP5224 at five two fold dilutions ranging in concentration from 0.0625ug to 1ug per spot. All five blots were hybridised with a biotin-labelled ICMP2668 DNA probe at 60^0C for 20hr. Blot 1 was developed with BSA buffers, blots 2, 3 and 4 were developed with a blocking buffer, complex dilution buffer or washing buffer which had milk powder substituted for BSA respectively and blot 5 was developed with milk powder in all buffers. In all blots there was no apparent difference in the intensity of colour produced, the limit of detection or the level of background.

3.1.5 The Precision of the LKB Scanning Densitometer

To determine the precision with which an LKB UltroScan XL scanning densitometer would read the colour intensity of developed slot blots three different hybridised and developed strips were read three times each with the same settings (section 2.2.142) on the scanning densitometer. The peak height minus the control peak height and the average peak height are shown in Table V. Not enough results were produced for statistical analysis, however, the results indicated that although there is variation in peak heights, particularly when higher values are involved, the values can be used to show general trends but are not precise enough to give an accurate value.

3.1.6 The repeatability of Results Obtained using a Biotin-labelled Probe

To determine how repeatable results of slot-blots made with total genomic ICMP2668 DNA and hybridised with an homologous biotin-labelled probe were, three identical slot-blot strips were hybridised at 60^0C for 20hr and developed under identical

conditions. The results were read on a scanning densitometer. The peak heights minus the peak height of the control (1ug *E.coli* DNA) and the average peak height are shown in Table VI. Although there are some quite marked variations in individual values the general trend is the same. Since the LKB scanning densitometer does not give very precise readings, the biotin-labelled probe gives reasonably reproducable results.

3.1.7 Determination of the Optimum Probe Concentration

To determine the optimum concentration of biotin-labelled probe DNA per ml of hybridisation buffer six identical slot blot strips were prepared with DNA from *E.coli* and *Rhizobium* strain ICMP2668. Each strip was hybridised with a different amount of biotin-labelled ICMP2668 DNA probe in 2.5ml of hybridisation buffer. The reaction was terminated when the control slot (1ug of *E. coli* DNA) had just begun to colour. The strips were read on the LKB scanning densitometer. The peak heights minus the height of the control peak are shown in Table VII. It is concluded that an increase in probe concentration gives a corresponding increase in the intensity of colour produced but does not increase the limit of detection. The minimum usable concentration with no loss in detection limit is 200ng/ml.

3.1.8 The Effect of Time on the Intensity of Colour Produced

To determine the relationship between the length of time that a hybridised slot blot is incubated with the reaction mixture and the intensity of colour produced seven identical slot-blot strips were prepared with DNA from *E.coli* and *Rhizobium* strain ICMP2668. All the strips were hybridised separately with a biotin-labelled ICMP2668 DNA probe at 60^0C for 20hr. Incubation with the reaction mixture was terminated at a different time for each strip. All the strips were read on a scanning densitometer and the resulting peak heights minus the control peak height (1ug *E.coli* DNA) are shown in Table VIII. There is a definite relationship between length of development time and the intensity of colour produced. Although the intensity of colour did not decrease after 7.5min the height of the control peak increased, so when the value for the control peak was subtracted from the value for the sample peak the

TABLE V : The Precision of Readings Obtained with an lkb Scanning Densitometer for Genomic DNA Slot-blots Hybridised with a Homologous Biotin-labelled DNA Probe at 60°C for 20hr and Visualised with a Streptavidin/Alkaline Phosphatase Conjugate under Standard Conditions

	DNA (ug)	COLOUR INTENSITY (mm) READING NUMBER 1	2	3	MEAN
STRIP A	**1.000**	0.42	0.33	0.41	0.39
	0.500	0.25	0.22	0.25	0.24
	0.250	0.12	0.07	0.08	0.09
	0.125	0.04	0.02	0.03	0.03
STRIP B	**1.000**	0.13	0.11	0.13	0.12
	0.500	0.11	0.12	0.12	0.12
	0.250	0.04	0.03	0.04	0.04
	0.125	0.06	0.15	0.08	0.10
STRIP C	**1.000**	0.05	0.05	0.05	0.05
	0.500	0.00	0.01	0.01	0.01
	0.250	0.02	0.02	0.02	0.02
	0.125	0.01	0.01	0.01	0.02

TABLE VI : The Repeatability of Results from Genomic DNA Slot-blots Hybridised with a Homologous Biotin-labelled DNA Probe at 60°C for 20hr and Visualised with a Streptavidin/Alkaline Phosphatase Conjugate under Standard Conditions

DNA (ug)	COLOUR INTENSITY (mm) STRIP LETTER A	B	C	MEAN
1.000	0.13	0.12	0.13	0.127
0.500	0.11	0.05	0.08	0.080
0.250	0.04	0.06	0.05	0.050
0.125	0.06	0.04	0.03	0.043
0.063	0.00	0.01	0.01	

result decreased. From these results it was concluded that it is important to develop blots until the control is just starting to colour and no further as after this the non-specific reaction adversely affects the result.

3.1.9 The Effect of Temperature on Colour Development

To determine the effect of temperature on the intensity of colour produced during development three identical slot-blot strips were made with DNA from *E.coli* and *Rhizobium* strain ICMP2668. All three strips were hybridised separately with a biotin-labelled ICMP2668 probe at 60^0C for 20hr. All three strips were incubated with the reaction mixture for 8min, one at 37^0C, one at 27^0C and one at 4^0C. The strips were read on a scanning densitometer and the peak heights minus the control peak height (1ug *E.coli* DNA) are shown in Table IX. Temperature affects the rate of colour production because it affects the rate of enzyme activity. There are marked differences in colour intensity between 27^0C and 37^0C. This is within the range of temperatures found in the laboratory and this suggests that unless the blots are all developed at exactly the same temperature it would not be possible to make comparisons between blots developed seperately and that no specific time for development could be set.

3.1.10 The Effect of Dextran Sulphate on the Rate of Hybridisation

To determine the effect on the minimum hybridisation time of including 5% dextran sulphate in the hybridisation buffer four identical slot blot strips were made with DNA from *E.coli* and *Rhizobium* strain ICMP2668. The four strips were hybridised at 60^0C for different periods of time with a biotin-labelled ICMP2668 DNA probe in 2.5ml hybridisation buffer to which 5% dextran sulphate had been added. Once developed the strips were read on the LKB scanning densitometer. The peak heights minus the control peak height (1ug *E.coli* DNA) are shown in Table X. There is a marked increase in the amount of colour produced and therefore the amount of hybridisation that has occurred between 2 and 6 hours of hybridisation time in the presence of dextran sulphate, but after 6 hours hybridisation time there is little change in the colour intensity produced.

TABLE VII : The Effect of the Concentration of Probe DNA in the Hybridisation Buffer on the Intensity of Colour Produced on Genomic DNA Slot-blots Hybridised with a Homologous Biotin-labelled DNA Probe at 60°C for 20hr and Visualised with a Streptavidin/Alkaline Phosphatase Conjugate under Standard Development Conditions

DNA (ug)	COLOUR INTENSITY (mm) CONCENTRATION OF PROBE DNA IN HYBRIDISATION BUFFER (ng/ml) 48	70	150	200	250	300
1.000	0.04	0.15	0.19	0.17	0.19	0.24
0.500	0.02	0.04	0.09	0.23	0.14	0.00
0.250	0.02	0.01	0.02	0.07	0.06	0.12
0.125	0.01	0.02	0.01	0.00	0.02	0.04
0.063	0.00	0.02	0.01	0.00	0.00	0.00
0.031	0.00	0.01	0.00	0.00	0.00	0.00

TABLE VIII : The Effect of Development Time on the Intensity of the Colour Produced on Genomic DNA Slot-blots Hybridised with a Homologous Biotin-labelled DNA Probe at 60°C for 20hr and Visualised with a Streptavidin/Alkaline Phophatase Conjugate

	COLOUR INTENSITY (mm)						
DNA	DEVELOPMENT TIME (mins)						
(ug)	**3.0**	**4.4**	**6.0**	**7.5**	**9.0**	**11.0**	**13.0**
1.000	0.01	0.00	0.02	0.21	0.12	0.12	0.04
0.500	0.01	0.00	0.03	0.10	0.04	0.05	0.05
0.250	0.00	0.00	0.01	0.13	0.06	0.05	0.04
0.125	0.00	0.00	0.00	0.11	0.00	0.03	0.00
0.063	0.01	0.00	0.00	0.08	0.00	0.00	0.00

TABLE IX : The Effect of the Temperature of Incubation with the Enzyme Substrate on the Intensity of Colour Produced on Genomic DNA Slot-blots Hybridised with a Homologous Biotin-labelled DNA Probe at 60°C for 20hr and Visualised with a Streptavidin/Alkaline Phophatase Conjugate

DNA (ug)	COLOUR INTENSITY (mm) TEMPERATURE OF INCUBATION WITH ENZYME SUBSTRATE (°C) 37	27	4
1.000	0.42	0.13	0.05
0.500	0.25	0.11	0.00
0.250	0.12	0.04	0.02
0.125	0.04	0.06	0.01
0.063	0.01	0.00	0.00

An experiment to determine the minimum time of hybridisation and to compare this with hybridisations carried out in the absence of dextran sulphate was carried out. Six identical slot blots were made with DNA from *E.coli* and *Rhizobium* strain ICMP2668. Three strips where hybridised with a biotin-labelled ICMP2668 probe in hybridisation buffer containing 5% dextran sulphate at 60°C for 1, 2 and 3 hours respectively. The other three strips where hybridised with a biotin-labelled ICMP2668 probe in unmodified hybridisation buffer at 60°C for 2, 3 and 4 hours respectively. All six strips were read on the LKB scanning densitometer and the peak heights minus the control peak height (1ug *E.coli* DNA) are given in Table XI. There was a decrease in the colour produced in the samples that were hybridised with dextran sulphate for less than 3 hours. The production of colour in the sample hybridised without dextran sulphate for 4 hours is comparable with the sample hybridised with dextran sulphate for 2 hours. The use of dextran sulphate in the hybridisation buffer reduces the minimum hybridisation time possible to achieve readable results to 3 hours from 6 hours.

3.1.11 The Specificity of Biotin-Labelled DNA Probes and ^{32}P-labelled DNA Probes

To determine whether biotin-labelled probes where as specific as ^{32}P probes two identical slot blot strips where prepared with 1ug of DNA from *E.coli* and *Rhizobium* strains ICMP2668, Gal14, NZP5232, 505/1, NZP5531 and U45. One slot-blot was hybridised with a ^{32}P-labelled ICMP2668 DNA probe (figure 7) and the other slot-blot was hybridised with a biotin-labelled ICMP2668 DNA probe (figure 8). Both labels only gave a significant reaction with the the *Rhizobium leguminosarum* strains.

3.1.12 Limit of Detection of Genomic DNA with a Homologous Biotin-Labelled Probe

To determine the limit of detection of genomic DNA with an homologous genomic biotin-labelled probe and the repeatability of the results three slot-blot strips were made with DNA from *E.coli* and *Rhizobium* strain ICMP2668. All three strips were hybridised with a biotin-labelled ICMP2668 DNA probe at 60°C for 20hr and developed under identical conditions. The results were read on an LKB scanning

TABLE X :The Effect of the Presence of 5% Dextran Sulphate in the Hybridisation Buffer on the Intensity of Colour Produced on Genomic DNA Slot-blots Hybridised with a Homologous Biotin-labelled DNA Probe at 60°C and Visualised with a Streptavidin/Alkaline Phophatase Conjugate under Standard Development Conditions

DNA (ug)	COLOUR INTENSITY (mm) HYBRIDISATION TIME (hours) 2	4	6	8
1.000	0.06	0.12	0.18	0.20
0.500	0.02	0.05	0.09	0.08
0.250	0.03	0.06	0.05	0.04
0.125	0.00	0.04	0.00	0.02
0.063	0.00	0.01	0.00	0.00

TABLE XI : The Rate of Hybridization of a Biotin-labelled Genomic DNA Probe with a Homologous DNA Slot-blot at 60°C in Hybridisation Buffer with and without 5% Dextran Sulphate, Visualised with a Streptavidin/Alkaline Phophatase Conjugate under Standard Development Conditions

	COLOUR INTENSITY (mm)					
DNA (ug)	HYBRIDISATION TIME WITH DEXTRAN SULPHATE (hours)			HYBRIDISATION TIME WITHOUT DEXTRAN SULPHATE (hours)		
	1	**2**	**3**	**2**	**3**	**4**
1.000	0.05	0.07	0.13	0.04	0.06	0.08
0.500	0.04	0.05	0.08	0.04	0.02	0.07
0.250	0.03	0.04	0.05	0.02	0.03	0.04
0.125	0.03	0.02	0.03	0.00	0.02	0.02
0.063	0.02	0.01	0.01	0.00	0.00	0.01

Figure 7: An autoradiograph of a slot-blot containing genomic DNA from a various *Rhizobium* species hybridised with a ^{32}P-labelled genomic *Rhizobium leguminosarum* biovar *trifolii* strain ICMP2668 DNA probe at 60^{o}C for 20hr and visualised with a streptavidin/alkaline phosphatase conjugate.

The slots contain 1ug of DNA (in duplicate) from:

slot number	**DNA origin**
1	*Escherichia coli*
2	*Rhizobium leguminosarum* biovar *trifolii*
3	*Rhizobium* sp. *(Galiga)*
4	*Rhizobium leguminosarum* biovar *phaseoli*
5	*Rhizobium leguminosarum* biovar *leguminosarum*
6	*Bradyrhizobium japonicum*
7	*Rhizobium meliloti*
8	*Rhizobium fredii*

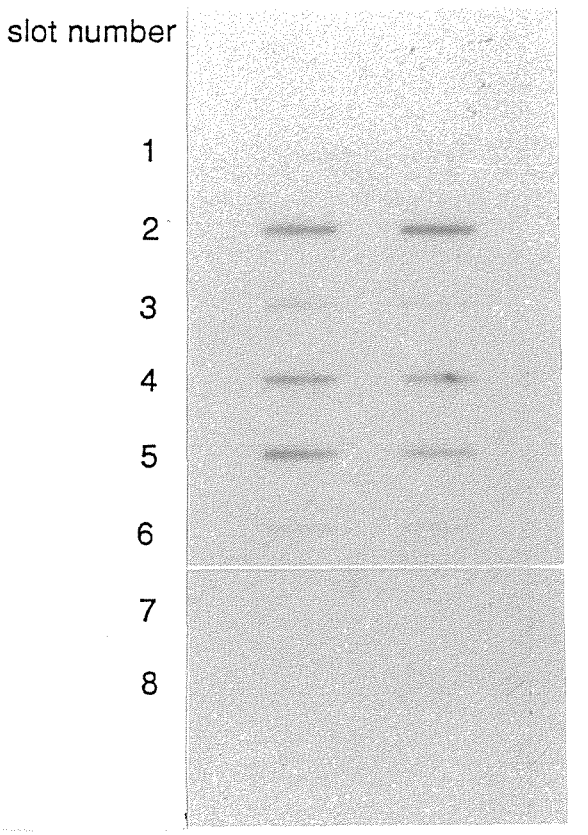
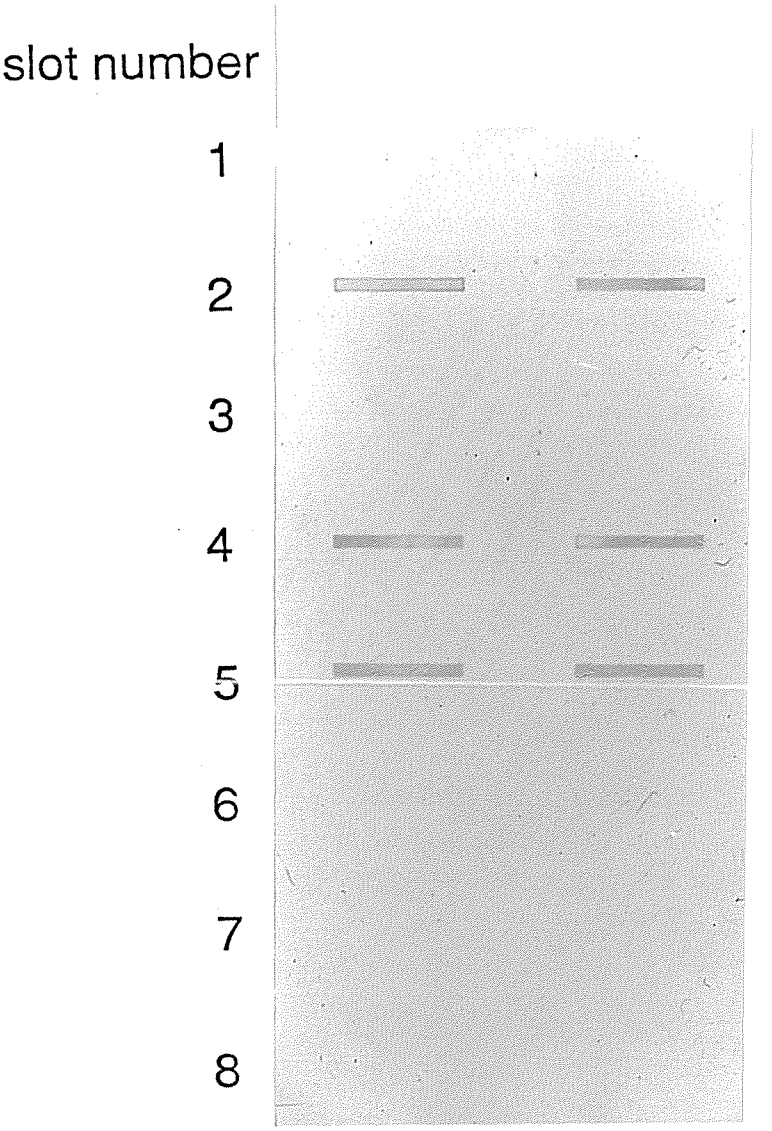

slot number
1
2
3
4
5
6
7
8

Figure 8: A slot-blot containing genomic DNA from various *Rhizobium* species hybridised with a biotin-labelled genomic *Rhizobium leguminosarum* biovar *trifolii* strain ICMP2668 DNA probe at 60°C for 20hr.

The slots contain 1ug of DNA (in duplicate) from:

slot number	**DNA origin**
1	*Escherichia coli*
2	*Rhizobium leguminosarum* biovar *trifolii*
3	*Rhizobium* sp. *(Galiga)*
4	*Rhizobium leguminosarum* biovar *phaseoli*
5	*Rhizobium leguminosarum* biovar *leguminosarum*
6	*Bradyrhizobium japonicum*
7	*Rhizobium meliloti*
8	*Rhizobium fredii*


slot number
1
2
3
4
5
6
7
8

densitometer and the peak heights minus the control peak height (1ug E.coli DNA) and the average peak height are given in Table XII. It is concluded that the limit of detection for total genomic *Rhizobium* DNA, using a slot-blot, with an homologous DNA biotin-labelled probe is between 0.063ng and 0.125ug.

3.1.13 Colony and Nodule Blots

To determine if it was possible to use a crude DNA extract as the target for hybridisation with a biotin-labelled DNA probe two colony-blots were made with *E. coli* and *Rhizobium* strains ICMP2668 and ICMP2668(RP4) colonies and two slot-blots where made with DNA from *E. coli*, *Rhizobium* strains ICMP2668and ICMP2668(RP4) and the RP4 plasmid. One colony-blot and one slot-blot were hybridised with a biotin-labelled ICMP2668 DNA probe while the other two were hybridised with a biotin-labelled RP4 plasmid DNA probe. The resulting colour development is shown in Table XIII. The colony blot shows no specificity but this is due to a non-specific reaction occurring on the colony-blot method and does not imply that the DNAs are indistinguishable since the DNA slot-blot differentiates between the strains successfully.

To try to reduce the lack of specificity, which may have been caused by the streptavidin-alkaline phosphatase conjugate binding to protein in the sample, a more through proteinase treatment which involved submerging the colony-blot in the proteinase K solution was compared with floating the colony-blot on the proteinase K solution. Four colony blots where made each containing an *E. coli* and *Rhizobium* strain ICMP2668 and ICMP2668(RP4) colony. Two of the blots were placed on filter paper saturated with the proteinase K solution while the other two were submerged in the proteinase K solution. One blot from each treatment was hybridised with a ICMP2668 biotin-labelled probe while the other two were hybridised with an RP4 biotin-labelled probe. Altering the proteinase K treatment did not eliminate the non-specific binding of the streptavidin-alkaline phosphatase conjugate.

TABLE XII : Genomic DNA Slot-blots Hybridised with a Homologous Biotin-labelled DNA Probe at 60°C for 20hr and Visualised with a Streptavidin/Alkaline Phophatase Conjugate under Standard Development Conditions to Determine the Limit of Detection

	COLOUR INTENSITY (mm)			
DNA		STRIP		
(ug)	**A**	**B**	**C**	MEAN
1.000	0.13	0.12	0.13	0.127
0.500	0.11	0.05	0.08	0.080
0.250	0.04	0.06	0.05	0.050
0.125	0.06	0.04	0.03	0.043
0.063	0.00	0.01	0.01	

TABLE XIII : Hybridization of Colony-blots and Slot-blots Containing Genomic DNA from *Rhizobium leguminosarum* biovar *trifolii* strain ICMP2668, strain ICMP2668(RP4) and Plasmid RP4 DNA with Biotin-labelled *Rhizobium leguminosarum* biovar *trifolii* strain ICMP2668 DNA and Biotin-labelled RP4 Plasmid DNA Probes at 60°C for 20hr, Visualised with a Streptavidin/Alkaline Phophatase Conjugate under Standard Development Conditions

SOURCE OF MEMBRANE-BOUND DNA		INTENSITY OF HYBRIDISATION WITH BIOTIN-LABELLED DNA PROBES	
		ICMP2668	**RP4**
Colonies of :	***E.coli***	+	+
	ICMP2668	+	+
	ICMP2668(RP4)	+	+
DNA from :	***E.coli***	-	-
	ICMP2668	++	+
	ICMP2668(RP4)	+	+
	RP4	-	++

- = absence of colour; + = some colour; ++ = intense colour.

3.2 ISOLATION OF A SEQUENCE SPECIFIC FOR HOMOLOGY GROUP I OF *BRADYRHIZOBIUM* sp. (*LOTUS*)

Bradyrhizobium sp. *(Lotus)* has been shown to contain two distinct homology groups as determined by DNA reassociation experiments (figure I). The strains used were divided into two homology groups as follows:

Homology Group I: NZP2089, NZP2308, NZP2307=CC814S, NZP2021, NZP2141, NZP2087 and ATCC10325,
Homology Group II: NZP2228, NZP2192, NZP2076, NZP2201, NZP2243, NZP2178 and NZP2257.

To isolate a DNA sequence that was specific for homology group I a gene library of strain cc814S DNA (homology group I) was probed with cc814S genomic DNA that had undergone a subtraction hybridisation with DNA from a representative strain of homology group II, NZP2076. The clones that hybridised were subcultured and underwent further screening to determine whether they contained strain or group specific sequences.

3.2.1 Isolation of Cosmids Containing Sequences Specific for *Bradyrhizobium* sp. *(Lotus)* Homology Group I from a Gene Library of strain cc814S

The suspension of cells representing the gene library contained 6.6 x 10^9 colony forming units per ml. The suspension was diluted and plated out on large (13.5 cm diameter) LB^{TC} agar plates to give no less than 300 colonies per plate. Three plates were required to give a 95% probability of including any sequence of DNA in the library (section 2.2.19). The plates were incubated overnight at 37°C before the colonies were lifted onto sterile nitrocellulose membranes, lysed, neutralised and baked (section 2.2.20). The plates were reincubated for a further 24hr then stored at 4°C.

To obtain DNA sequences from *Bradyrhizobium* sp. *(Lotus)* strain cc814S that where not found in the *Bradyrhizobium* sp. *(Lotus)* strain NZP2076 genome 3ug of DNA from cc814S was digested with HAE111 in a 25ul reaction mixture (section 2.2.11). The mixture was boiled to dissociate the DNA then labelled with ^{32}P by random

priming (section 2.2.12). The labelled DNA was purified through a mini-spin column (section 2.2.13) to remove unincorporated ^{32}P. After passage through the mini-spin column 60% of the ^{32}P-deoxyCTP in the column effluent was incorporated in the DNA and the total radioactivity in the probe was 7.3 x 10^6 cpm. The column effluent was hybridised with 150ug of unlabelled NZP2076 DNA in 0.28M sodium phosphate buffer pH6.8 at 65^{o}C for 27hr. Single-stranded DNA specific for strain cc814S, was isolated from the double stranded non-specific DNA with hydroxylapatite (section 2.2.22). The first two washes of 2ml each contained 9 x 10^5 and 8 x 10^5 cpm/ml respectively. The third wash of 8ml contained 3 x 10^5 cpm/ml. The first two washes were pooled and recounted giving 8.7 x 10^5 cpm/ml. This was divided into three equal alliquots each containing 1.2 x 10^6 total cpm and hybridised to the three colony lift membranes at 65^{o}C for 24hr (section 2.2.14). The membranes were exposed to X-ray film (section 2.2.16) for 48hrs at -70^{o}C. The developed autoradiographs showed a faint reaction with all colonies but 8 colonies gave a more intense reaction and these were subcultured for further work. The number of colonies subcultured (14) exceeds the number of colonies that showed a positive reaction (8) when hybridised. This was because the autoradiograph could not be matched to the plate with sufficient accuracy to distinguish between closely spaced colonies. These clones where referred to as the JG100 series and numbered JG101 through to JG114 inclusively.

3.2.2 A Comparison of the Rapid Boil Method (section 2.2.23) and the Potassium Acetate Method (section 2.2.24) for the Rapid Isolation of Cosmids

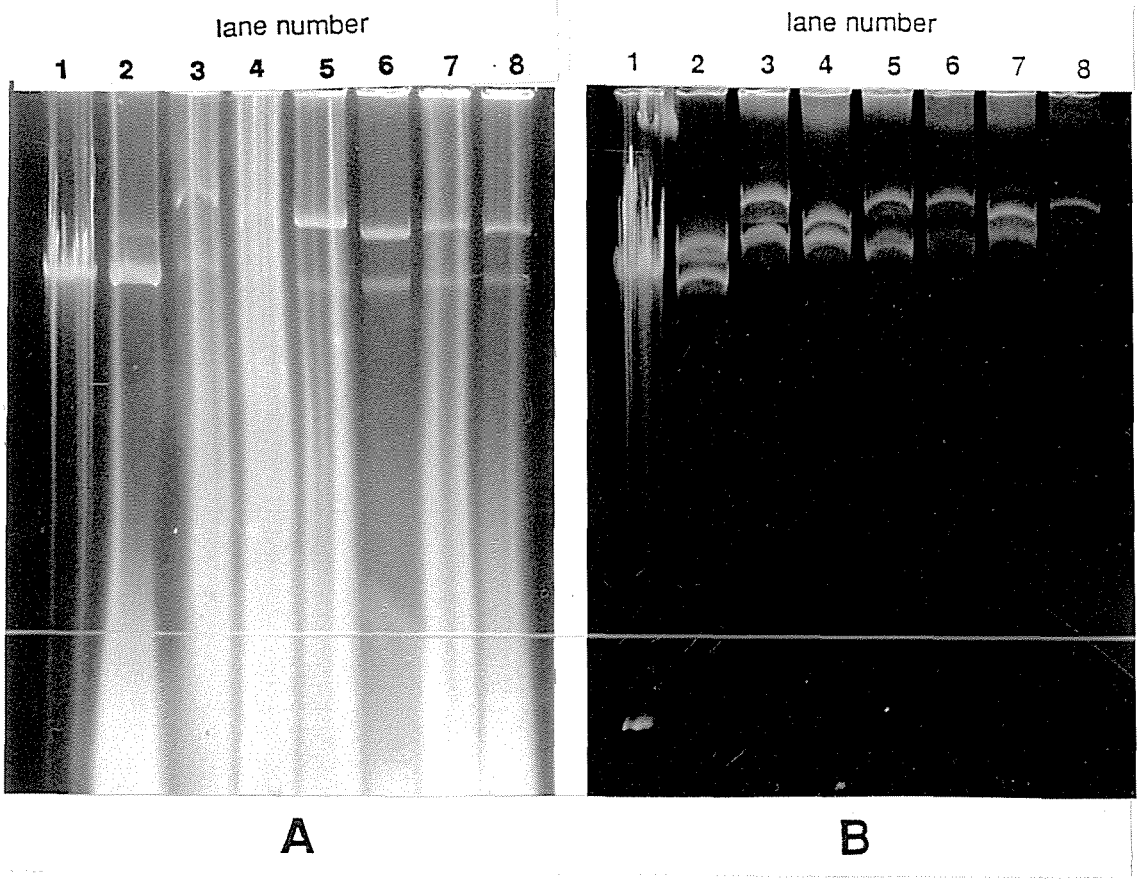
For secondary screening a method for rapid cosmid isolation was required. Two methods of cosmid isolation, rapid boil and potassium acetate precipatation, were compared to determine which method resulted in removing the most cell material to give the sharpest and clearest bands when separated by agarose gel electrophoresis. Clone isolates JG101 through to JG107 inclusive where each treated by both methods to isolate their cosmids. The preparations were separated by agarose gel electrophoreseis (section 2.2.7). The gels stained with ethidium bromide and visualised with UV light are shown in figure 9. The rapid boil method left a lot of RNA and residual material which resulted in a badly streaked gel in which some of the cosmid bands were difficult to see. The potassium acetate method gave a much clearer result. It was concluded that for the isolation of the cosmids from the cc814S gene library in pLAFR1 the potassium acetate precipitation method gave the best result.

Figure 9: Agarose gels of the rapid boil method and the potassium acetate precipitation method for the isolation of cosmids.

Gel A: cosmids isolated by the rapid boil method
Gel B: cosmids isolated by the potassium acetate method

The agarose gels lanes contain:

lane number	DNA origin
1	*Bradyrhizobium* sp. *(Lotus)* strain NZP2076
2	clone JG101 cosmid
3	clone JG102 cosmid
4	clone JG103 cosmid
5	clone JG104 cosmid
6	clone JG105 cosmid
7	clone JG106 cosmid
8	clone JG107 cosmid


lane number
1 2 3 4 5 6 7 8
lane number
1 2 3 4 5 6 7 8
A
B

3.2.3 The Specificity of Cosmid Isolates Selected by Colony Hybridisation

To ensure that the cosmids containing putative strain-specific cc814S DNA inserts did not hybridise with the subtractor DNA from strain NZP2076, cosmid DNA from each of the fourteen clones was isolated using the potassium acetate precipatation method (section 2.2.24) and separated by agarose gel electrophoresis (section 2.2.7). One lane contained total genomic DNA from strain NZP2076 as a positive control. Southern blots (section 2.2.25) were hybridised at 65°C for 24hr with an ^{32}P-labelled genomic strain NZP2076 DNA probe. Cosmids JG102, JG104, JG108, JG110, JG111 and JG113 contained DNA which hybridised with the NZP2076 probe DNA. The remaining clones (JG101, JG103, JG105, JG106, JG107, JG109, JG112 and JG114) did not hybridise indicating that they contained strain cc814S DNA sequences that were not found in strain NZP2076 (figure 10). These clones underwent secondary screening to determine if they contained homology group-specific DNA sequences.

Secondary screening to determine the specificity of the selected clones was carried out using eight slot-blots containing DNA (1ug in duplicate) from the following eight sources: *Bradyrhizobium* sp. *(Lotus)* strains NZP2076, NZP2192, NZP2257, NZP2021, the cosmid clone, cc814S, NZP2089 and NZP2141. Each slot blot was hybridised at 60°C for 24hrs with ^{32}P-labelled DNA from the cosmid included as the positive control (isolated by potassium acetate precipitation and further purified by phenol/chloroform extraction and ethanol precipitation). None of the clones hybridised with DNA from strain NZP2076, but all showed varying degrees of homology with each of the other strains of *Bradyrhizobium* tested (for an example see figure 11) and were therefore able to distinguish between strain NZP2076 and the other strains but lacked sufficent specificity to distinguish between the two *Bradyrhizobium* sp. *(Lotus)* homology groups.

3.2.4 Experiments to Increase the Selection for Sequences Specific for Homology Group I in the cc814S Gene Library

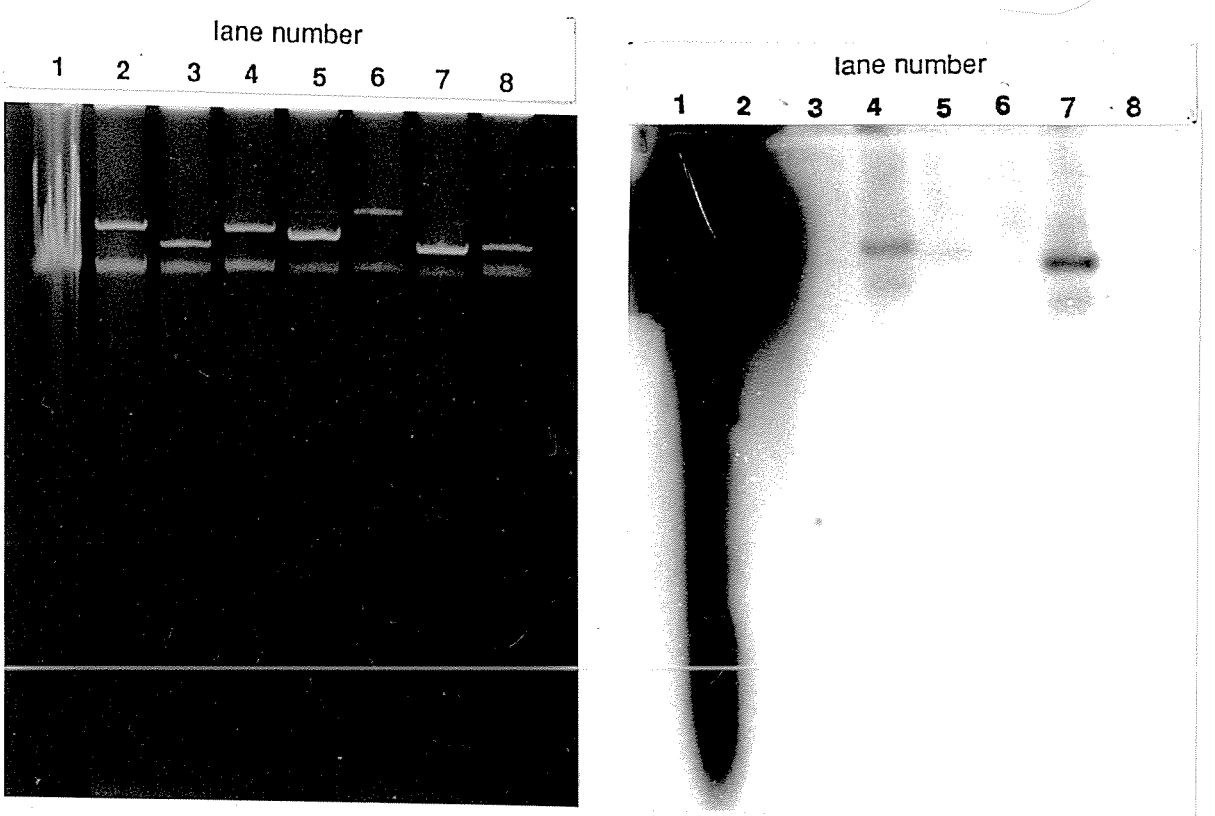
To retrieve sequences of the cc814S genome that where specific for homology group I, strain NZP2021 DNA (selected randomly from homology group I) was used in place of cc814S DNA to be treated with subtractor DNA. The ^{32}P-labelled NZP2021 DNA (5.3 x 10^6 total counts per minute, 92% incorporation) underwent a subtraction hybridisation with NZP2076 DNA and the unhybridised sequences were separated

Figure 10: Clones isolated from a gene library of *Bradyrhizobium* sp. *(Lotus)* strain cc814S (with DNA from strain cc814S that had undergone a subtraction hybridisation with DNA from *Bradyrhizobium* sp. *(Lotus)* strain NZP2076) screened to determine which contain sequences not found in *Bradyrhizobium* sp. *(Lotus)* strain NZP2076.

A: agarose gel from which southern blot B was taken
B: autoradiograph of southern blot of A hybridised with an NZP2076 probe

The lanes contain:

lane number	**DNA origin**
1	*Bradyrhizobium* sp. *(Lotus)* strain NZP2076
2	clone JG101 cosmid
3	clone JG102 cosmid
4	clone JG103 cosmid
5	clone JG104 cosmid
6	clone JG105 cosmid
7	clone JG106 cosmid
8	clone JG107 cosmid



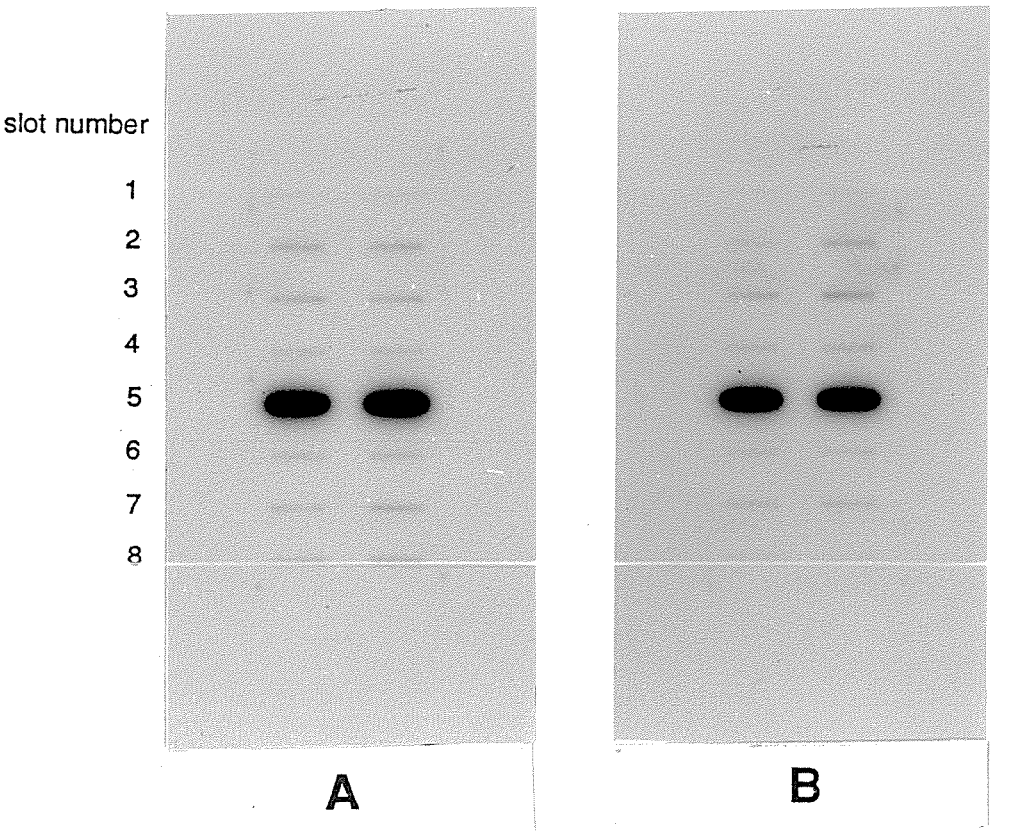
A B

Figure 11: Autoradiographs of slot-blots containing genomic DNA from *Bradyrhizobium* sp. *(Lotus)* strains from homology group I and homology group II hybridised with a ^{32}P-labelled cosmid DNA probe from a gene library of *Bradyrhizobium* sp. *(Lotus)* strain cc814S (with DNA from strain cc814S that had undergone a subtraction hybridisation with DNA from *Bradyrhizobium* sp. *(Lotus)* strain NZP2076) at 65^{o}C for 20hr.

A: hybridised with a clone JG109 cosmid probe
B: hybridised with a clone JG110 cosmid probe

The slot blots contain 1ug of DNA (in duplicate slots) from:

slot number	**DNA origin**
1	*Bradyrhizobium* sp. *(Lotus)* strain NZP2076
2	*Bradyrhizobium* sp. *(Lotus)* strain NZP2192
3	*Bradyrhizobium* sp. *(Lotus)* strain NZP2257
4	*Bradyrhizobium* sp. *(Lotus)* strain NZP2021
5	cosmid isolate
6	*Bradyrhizobium* sp. *(Lotus)* strain cc814S
7	*Bradyrhizobium* sp. *(Lotus)* strain NZP2089
8	*Bradyrhizobium* sp. *(Lotus)* strain NZP2141


slot number
1
2
3
4
5
6
7
8
A
B

from the hybridised sequences with hydroxylapatite. The first four washes of the hydroxylapatite contained 1 x 10^6, 8.3 x 10^5, 2.7 x 10^5 and 2.3 x 10^5 total counts per minute respectively. The last four washes contained 1.6 x 10^6, 8.0 x 10^5, 2.3 x 10^5 and 2.1 x 10^5 total counts per minute respectively. These results indicate that there was approximately 50% homology between strains NZP2021 and NZP2076. The first two washes were pooled and recounted to give specific labelled DNA (4ml) containing 2.2 x 10^6 cpm.

In all other ways this experiment was the same as the first. There were 8 positive clones and 14 clones were selected to ensure that the correct clones were chosen. These clones were referred to as the JG200 series, numbered JG201 through to JG214 inclusively. When screened to determine which were the correct isolates cosmids JG201, JG202, JG203, JG204, JG212 and JG214 contained DNA which hybridised with the NZP2076 probe DNA. The remaining clones (JG205, JG206, JG207, JG208, JG209, JG210, JG211 and JG213) did not hybridise indicating that they contained strain cc814S DNA sequences that where not found in strain NZP2076. These clones underwent secondary screening tests. None of the clones hybridised with DNA from strain NZP2076, but all showed varying degrees of homology with each of the other strains of *Bradyrhizobium* tested and therefore lacked sufficent specificity to distinguish between the two homology groups. However, isolates JG207 and JG210 gave a more intense reaction with strains from homology group I than with strains from homology group II (figure 12 A and B) indicating that while they contained sequences common to both groups some of their sequence may be specific for homology group I.

3.2.5 Subcloning Isolate JG210 Cosmid Fragments to try and Isolate a Smaller Sequence that was Specific for Homology Group I

To determine if any part of the DNA sequence in the cosmid from isolate JG210 was specific for homology group I the cosmid was purified on a cesium chloride gradient (section 2.2.26) and digested with endonuclease Sau3A (section 2.2.27) to give fragments of 400-600 base pairs. The vector pGEM2 was digested with endonuclease BAMH1 (section 2.2.27) and treated with calf alkaline phosphatase (CAP), to stop the vector from religating to itself, (section 2.2.28) and ligated with the JG210 fragments (section 2.2.29). The ligation mixture was visualised by agarose gel electrophoresis (section 2.2.7) all visible DNA had ligated (see figure 13).

Figure 12: Autoradiographs of slot-blots containing genomic DNA from *Bradyrhizobium* sp. *(Lotus)* strains from homology group I and homology group II hybridised with a ^{32}P-labelled cosmid DNA from a gene library of *Bradyrhizobium* sp. *(Lotus)* strain cc814S (with DNA from strain cc814S that had undergone a subtraction hybridisation with DNA from *Bradyrhizobium* sp. *(Lotus)* strain NZP2076) probe at 65^{o}C for 20hr.

A: hybridised with a clone JG207 cosmid probe
B: hybridised with a clone JG210 cosmid probe
C: hybridised with a clone JG209 cosmid probe

The slot blots contain 1ug of DNA (in duplicate slots) from:

slot number	**DNA origin**
1	*Bradyrhizobium* sp. *(Lotus)* strain NZP2076
2	*Bradyrhizobium* sp. *(Lotus)* strain NZP2192
3	*Bradyrhizobium* sp. *(Lotus)* strain NZP2257
4	*Bradyrhizobium* sp. *(Lotus)* strain NZP2021
5	cosmid isolate
6	*Bradyrhizobium* sp. *(Lotus)* strain cc814S
7	*Bradyrhizobium* sp. *(Lotus)* strain NZP2089
8	*Bradyrhizobium* sp. *(Lotus)* strain NZP2141

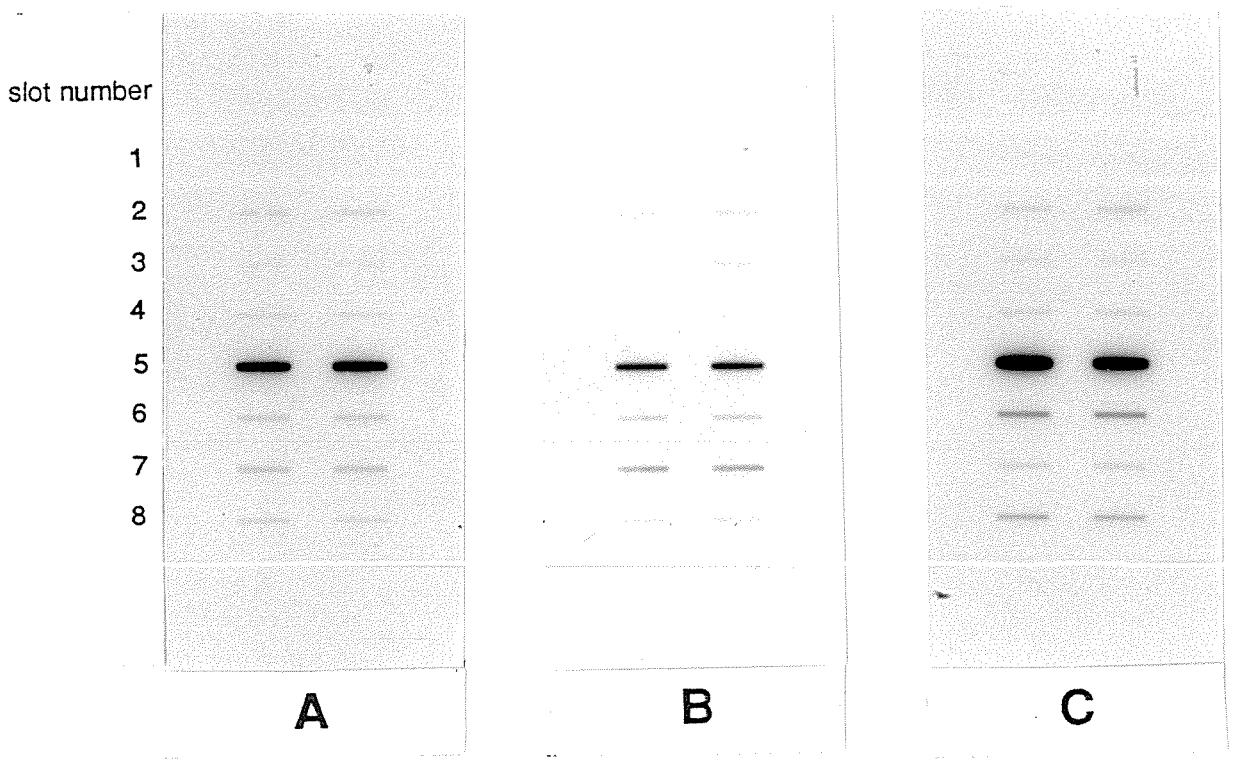
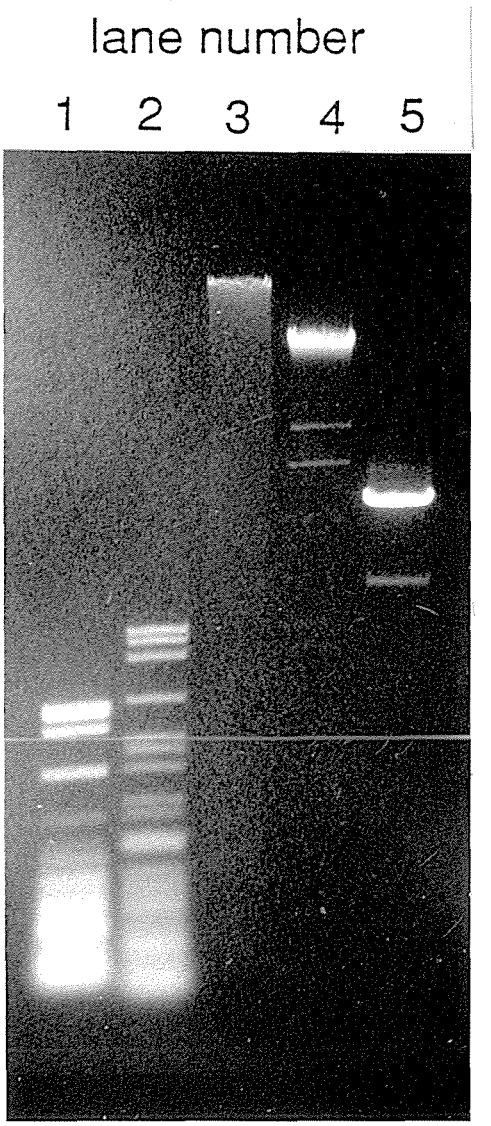

slot number
1
2
3
4
5
6
7
8
A
B
C

Figure 13: An agarose gel showing the result of a ligation

The lanes contain:

lane number	contents
1	JG210 cosmid digested with Sau3A
2	lambda digested with MSP2
3	ligation of JG210 cosmid (Sau3A) and pGEM2 (BamH1)
4	lambda digested with ECOR1
5	pGEM2 digested with BamH1


lane number
1 2 3 4 5

Electroporation (section 2.2.30) was used to transform ligated pGEM2 containing cloned DNA from the JG210 cosmid into *E.coli* strain HB101 cells. The transformed cells were spread on LB^{AMP} agar (section 2.2.1) and incubated for 24hrs at 37°C. The resulting colonies where blotted onto nitrocellulose and probed with a ^{32}P-labelled JG210 cosmid. Of these colonies 93% contained inserts. Two hundred and eighty eight of these where subcultured onto fresh LB^{AMP} agar plates to represent the cosmid library.

3.2.6 Screening the Cosmid Library for Homology Group I Specific Sequences

A duplicate of the representative colonies of the JG210 cosmid library was made. The representative library consisted of two 13.5cm diameter LB^{AMP} agar plates containing 144 colonies in a 12 x 12 grid. The libraries were blotted (section 2.2.20) and one set of blots was probed with ^{32}P-labelled NZP2089 (homology group I) and the other set of blots was probed with ^{32}P-labelled NZP2228 (homology group II). The resulting autoradiographs did not give a clear enough result to determine which colonies hybridised with one but not the other probe. This is a necessary prerequisite to isolation of sequences specific for homology group I. An experimental protocol which gives better resolution is required. This might be achieved by more stringent hybridisation and washing conditions but it could require a protocol that more thoroughly removes the cell constituents from colony lifts. I believe that these constituents may have caused a faint reaction to occur with all colonies regardless of whether they contained homologous DNA.

DISCUSSION

4.1 IDENTIFICATION OF *RHIZOBIUM* AND *BRADYRHIZOBIUM* SPECIES BY HYBRIDISATION OF A BIOTIN-LABELLED GENOMIC DNA PROBE TO TOTAL DNA BOUND TO NITROCELLULOSE

4.1.1 The use of Colony/Nodule Blots, Dot-blots and Slot-blots

The biotin-streptavidin/alkaline phosphatase detection system was applied to hybridisations of genomic DNA with crude colony and nodule extracts in the form of colony-blots and nodule-blots and to purified DNA in the form of dot-blots and slot-blots all on nitrocellulose membranes.

4.1.11 Nodule and Colony Blots

The extraction of DNA requires time, effort and expense so crude samples have often been probed directly. In the case of *Rhizobium* this has normally been in the form of nodule or colony blots (Hodgson, 1983; Cooper, 1987). While crude samples can be hybridised with ^{32}P-labelled probes using the method specified by Grunstein and Hogness (1975) the streptavidin-alkaline phosphatase tended to react non-specifically with impurities in the sample (probably endogenous biotin or avidin binding proteins in the cells) (Table XIII). Haas and Fleming (1986) modified this method to the form shown in Table XIV for use with non-radioactive probes to obtain satisfactory results with *Escherichia coli* and *Xanthomonas* colonies. When used on colonies of soil isolates a weak non-specific signal was present (Zeph, 1989). The DNA used in this study required purification by proteinase treatment, phenol extraction and ethanol precipitation to ensure that no non-specific binding occurred. Zwadyk (1986) found that DNA purified by electrophoresis was required to obtain satisfactory results.

4.1.12 A Comparison of the Dot-Blot and Slot-Blot Formats

Initial work with purified DNA was carried out by placing spots of DNA solution onto nitrocellulose (a dot-blot) but this made the analysis of results difficult as the

TABLE XIV : A Method for Producing Colony-blots for Hybridisation with Non-radioactive Probes

STEP NUMBER	PROCEDURE
1	7min wash in 0.5M NaOH
2	7min wash in 0.05M TrisHCl, pH8.0
3	3 x 1 min washes in 0.05M TrisHCl, pH8.0 (ice cold)
4	10min wash in lysozyme solution (1.5 mg/ml, 25% sucrose, 0.05M TrisHCl pH8.0, ice cold)
5	2 x 2 washes in 1xSSC at 37^{o}C
6	1hr wash in 200 ug/ml proteinase K in 1xSSC
7	2 x 1 min washes in 1xSSC
8	2 x 2 min washes in 90% ethanol
9	air dry
10	30min wash in phenol/chloroform/isoamylalcohol
11	2 x 10 min washes in chloroform
12	100ml chloroform through the filter by aspiration
13	air dry
14	bake for 2hr at 80^{o}C

size of the spot depended on how quickly the DNA solution adsorbed onto the nitrocellulose membrane (due to variability in the membrane). This variation in the concentration of DNA on the nitrocellulose membrane resulted in different intensities of colour being produced for the same DNA solution. To overcome the subsequent interpretation problems associated with this phenomena a slot-blot apparatus (Bio-Rad, Figure 3) was used. This allowed the application of the DNA solution to the membrane over a constant area and also had the advantage in that the slot-blot strips, when developed, could be given a numerical value with a scanning densitometer. Figure 14 shows a dot-blot and a slot-blot. The dot-blot shows the uneven spot sizes, the difference was greater than this in other membranes. The slot-blot while giving a constant area of deposition sometimes did not show an even distribution of colour and hence DNA throughout the slot.

4.1.13 Effect of Ethanol on the Quality of Nitrocellulose Dot-Blots

To obtain a dot-blot that is easy to interpret and photograph it is desirable for the membrane to remain flat throughout the procedure. In the protocol used for the production of dot-blots (section 2.2.8) the membrane was rinsed with ethanol under vacuum to dissolve and remove any material that might produce a non-specific reaction with the streptavidin-alkaline phosphatase complex, however the use of ethanol on nitrocellulose caused permanent wrinkling. The ethanol wash was successfully omitted with no apparent change in the intensity of the colour produced, the detection limit or the level of background (section 3.1.3).

4.1.14 The use of the LKB Scanning Densitometer

A scanning densitometer was used to give a numerical value to the colour intensity of the slots on a slot-blot membrane. The densitometer gave a print out which showed peaks corresponding to the presence of colour on the filter. Each peak was given the values of height (mm), area (mm^2) and relative area as a percentage of the area under the graph. The base line did not remain level between the major peaks, which correspond to the slots, because the machine recorded many minor (between band) peaks. This affected the value of the area and hence the value of the relative area producing inaccurate results. Figure 5 shows an example of a scan of a good slot-blot however, figure 15 is more typical of the results achieved. Due to the above problems

Figure 14: An example of a slot-blot and a dot-blot.

1 : The six slot-blot strips contain DNA from *Escherichia coli* in slot 1 and from *Rhizobium leguminosarum* biovar *trifolii* strain ICMP2668 in slots 2 through to 8 in the following concentrations:

slot number	DNA (ug/slot)
1	1.000
2	1.000
3	0.500
4	0.250
5	0.125
6	0.063
7	0.031
8	0.016

Each strip was hybridised with a biotin-labelled *Rhizobium leguminosarum* biovar *trifolii* strain ICMP2668 DNA probe at 60°C for 20hr and visualised with a streptavidin/alkaline phosphatase conjugate. The concentration of the probe DNA in the hybridisation buffer was as follows:

strip	probe concentration (ng/ml)
A	48
B	70
C	150
D	200
E	250
F	300

2 : A dot-blot containing genomic DNA in columns (left to right) from *Escherichia coli*, *Rhizobium leguminosarum* biovar *trifolii* strain ICMP2668, *Rhizobium leguminosarum* biovar *trifolii* strain 505/1, *Rhizobium leguminosarum* biovar *leguminosarum* strain NZP5224 and *Rhizobium leguminosarum* biovar *phaseoli* strain NZP5232 at concentrations (top to bottom) of 1ug, 0.5ug, 0.25ug and 0.125ug per spot hybridised with a biotin-labelled genomic *Rhizobium leguminosarum* biovar *trifolii* strain ICMP2668 DNA probe and visualised with a streptavidin/alkaline phosphatase conjugate.

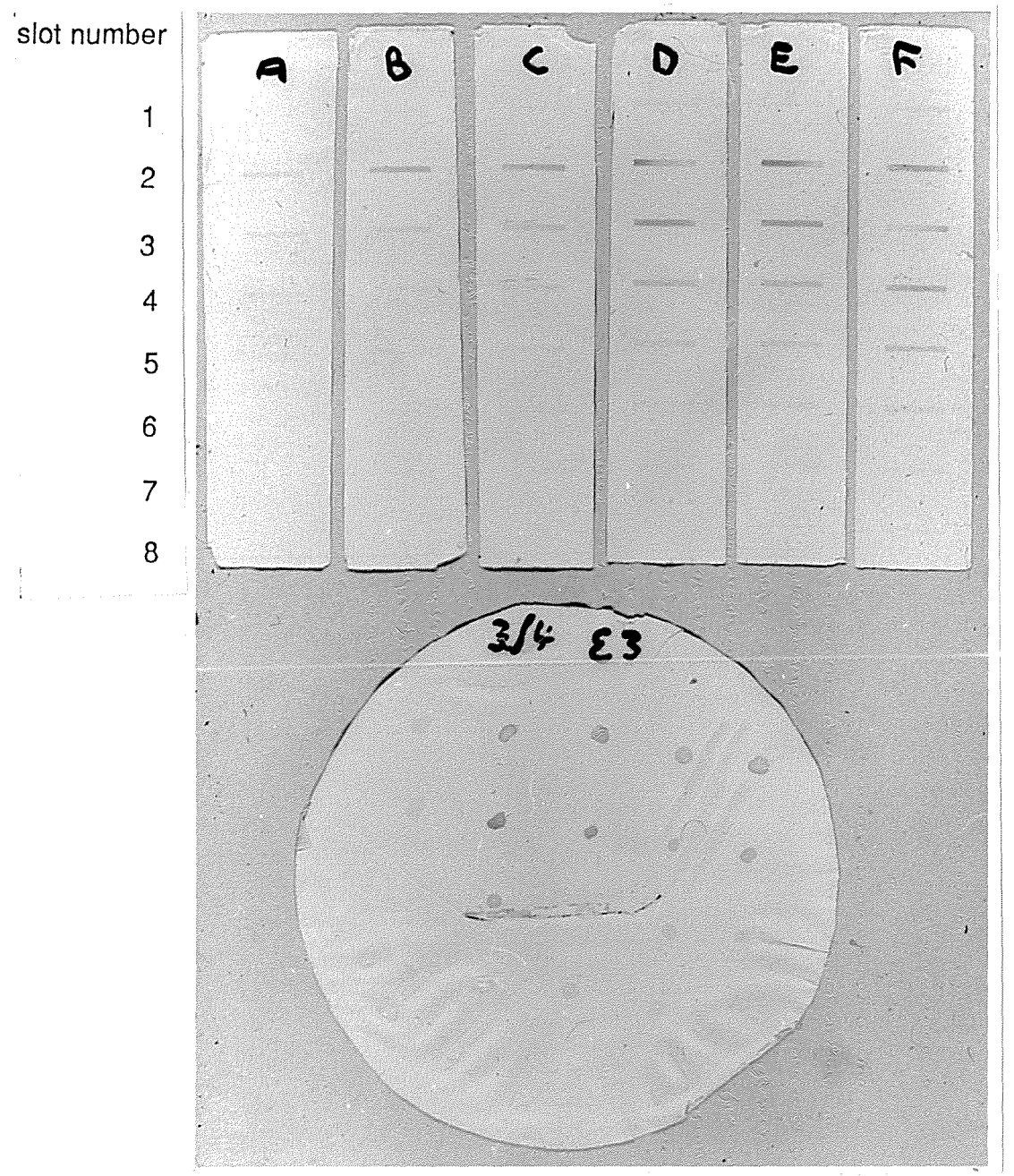

slot number
1
2
3
4
5
6
7
8
A
B
C
D
E
F
3/4 E3

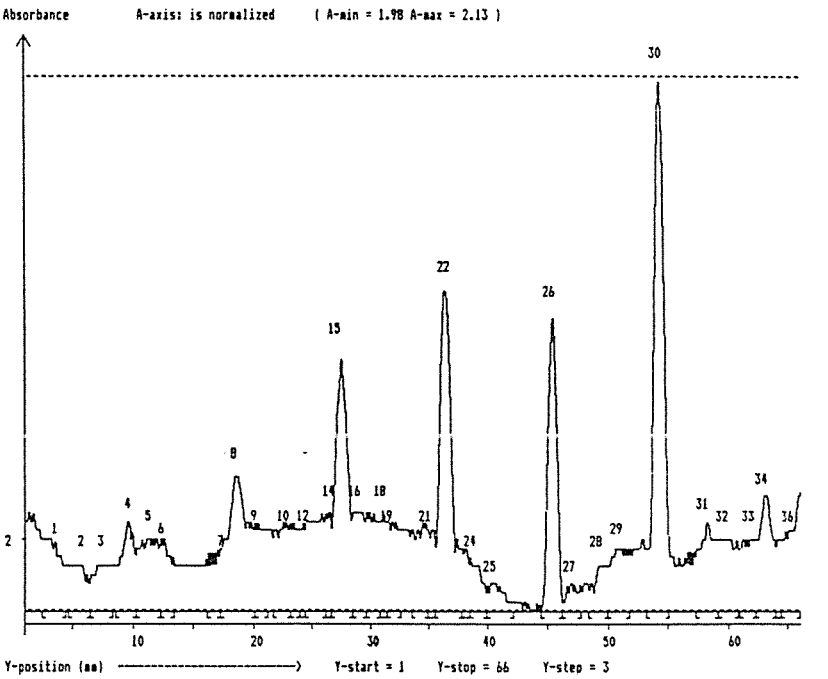
Figure 15: An example of a print out from the LKB scanning densitometer of a slot-blot containing genomic *Rhizobium leguminosarum* biovar *trifolii* strain ICMP2668 DNA hybridised at 60°C for 20hr with a homologous biotin-labelled probe and visualised with a steptavidin/alkaline phosphatase conjugate under standard development conditions.

peak	DNA	ug
34	*E.coli*	1.000
30	ICMP2668	1.000
26	ICMP2668	0.500
22	ICMP2668	0.250
15	ICMP2668	0.125
08	ICMP2668	0.063

LKB UltroScan XL

Run by Operator: £ 17 Run ID £ = 2319 Tuesday Dec-22-1987 14:29

Track = 1 of 9 X-position = 20.0 X-width = 6
Type of beam = line Smoothing: YES Peak-width = 2
Base line: at 1.98 AU is average of the 16 lowest data points (type = 1)



PEAK £	POSITION mm	HEIGHT AU	AREA AU*mm	REL. AREA %	PEAK £	POSITION mm	HEIGHT AU	AREA AU*mm	REL. AREA %
1	3.5	0.02	0.03	2.7	19	31.7	0.02	0.03	2.4
2	5.6	0.01	0.02	1.6	20	34.6	0.02	0.03	2.5
3	7.4	0.01	0.02	1.8	21	35.0	0.02	0.01	1.2
4	9.6	0.02	0.03	2.5	22	36.4	0.09	0.11	8.3
5	11.3	0.02	0.04	3.1	23	37.9	0.01	0.01	1.0
6	12.5	0.02	0.02	1.5	24	39.0	0.01	0.02	1.2
7	17.2	0.01	0.02	1.4	25	40.4	0.01	0.01	0.9
8	18.6	0.04	0.08	6.0	26	45.4	0.08	0.07	5.3
9	20.3	0.02	0.03	2.1	27	46.8	0.01	0.01	0.7
10	22.7	0.02	0.03	2.7	28	49.3	0.01	0.02	1.3
11	23.3	0.02	0.02	1.7	29	50.8	0.02	0.03	2.3
12	24.4	0.02	0.01	1.1	30	54.1	0.15	0.14	11.3
13	25.8	0.03	0.04	3.5	31	58.2	0.02	0.04	3.1
14	26.4	0.03	0.02	1.2	32	59.9	0.02	0.03	2.6
15	27.5	0.07	0.08	6.6	33	61.9	0.02	0.03	2.1
16	29.0	0.03	0.03	2.7	34	63.0	0.03	0.04	3.4
17	30.2	0.03	0.03	2.6	35	64.2	0.02	0.01	0.9
18	31.1	0.03	0.02	1.4	36	65.0	0.02	0.04	3.2

only the value for peak height was used as a measure of colour intensity as this was unaffected by the presence of minor peaks. A problem encountered with the scanning of slot-blots was that in some slots the deposition of the colour was not even across the slot (Figure 14) which resulted in a low value being given if the scanning line passed through a pale area (*eg* figure 15, peak 26) and a high value if the scanning line passed through a dark area. To try and combat this the scanning line was set to the widest possible value without introducing the possibility of reading beyond the end of the slot. Some anomalies still occurred and this combined with the lack of precision in the readings, especially where higher values were concerned, (section 3.1.5) and the limited repeatability of results (section 3.1.6) meant that the results could only be used to show general trends and not as accurate measurements. Blots developed on different days showed different intensities so it was not possible to make comparisons between blots that had not been developed concurrently.

4.1.2 Modification of the Method to Obtain Optimum Results

When an enzyme based hybridisation system is used the enzyme reaction must be controlled and the effect of variable conditions understood so that the optimum parameters for a particular application can be set. These tend to vary depending on the probe and target DNA used. Modifications were also made to reduce cost and hybridisation time.

4.1.21 Control over the Termination of the Colour Reaction

Since the production of pigment is catalysed by alkaline phosphatase, pigment will continue to be produced until conditions become unfavorable for enzyme activity. If left to dry enzyme activity continues until the membrane is completely dry so that if the membrane is just removed from the substrate after the desired intensity of colour has been obtained it subsequently becomes overdeveloped, *ie* pigment is produced in the negative control. Samples that are overdeveloped show an increase in colour intensity in both the samples and the negative control. As this is not proportional, the real value (sample peak height minus negative control peak height) shows a marked decrease (Table VIII). It is therefore desirable to be able to stop the colour reaction as soon as the negative control begins to colour. Rapid dehydration did not stop the enzyme action immediately and gave the same result as if the membrane had been air

dried. When the filter was rinsed in TE Buffer (Leary, 1983) the enzyme was inhibited by EDTA which along with the dilution and removal of substrate stopped the production of pigment immediately. After rinsing the filter can be left to air dry and no further pigment production occurs (Table IV). This is the method predominantly used to terminate the enzyme reaction (Renz, 1984; Gebeyuhu, 1987; Zeph, 1989)

4.1.22 The Effect of Various Parameters on Colour Production

Using the scanning densitometer to give a numerical value for the slot-blot strips it was possible to determine the effect of various parameters on the intensity of colour produced.

4.1.221 Probe Concentration

A higher concentration of probe DNA resulted in a more intense colour being produced but the limit of detection was unaffected (Table VII) so that if a probe needed to be conserved due to scarcity of DNA or expense considerations then a smaller amount could be used without decreasing the limit of detection. The minimum amount was 200ng/ml which is within the limits of reported concentrations. While it has been reported that high concentrations of probes, in the order of 100 - 1000ng/ml, give an unacceptable level of background with 20ng/ml being a more suitable concentration (Forster, 1985) it has also been reported that virtually no non-specific reactions were seen at concentrations up to 750 ng/ml (Leary, 1983). The reported concentrations of probe DNA in the hybridisation buffer used vary greatly: 20 - 75 ng/ml (Chan, 1985); 50 - 100 ng/ml (Gebeyuhu, 1987; Zeph, 1989); 200 ng/ml (BRL); 250 ng/ml (Imaeda, 1988). This indicates that the determining factors in the concentration of probe required are the size of the probe sequence and the size, concentration and presentation of the target sequence. Different applications require different probe concentrations, and these require individual assesment.

4.1.222 Temperature

As a major component of this detection system is an enzymatic reaction the temperature at which the blot is incubated with the substrate will affect the rate of colour deposition. Higher temperatures of incubation result in more rapid pigment

production. The enzyme system is particularly sensitive over the range of temperatures found in the laboratory (approximately 15^0C - 30^0C) so that minor fluctuations in temperature gave marked differences in development time (section 3.1.9). There are no reports of streptavidin/alkaline phosphatase detection systems being developed at anything other than room temperature, and the reported development times span 20min (Haas, 1986) to 16-20hr (Chan, 1985) so temperature is not the critical factor affecting development time.

4.1.223 Development time

It was not possible to set a standard development time as this was affected by unknown variables. Development time varied from 5 to 30 minutes so development was always continued until the negative control began to show colour. In most cases development times reported in the literature are considerably longer: 1 - 3 hr, first visible at 30min (Gebeyuhu, 1987); 2hr (Detek) (Imaeda, 1988); 3hrs (BRL); 3 - 4 hr (Leary, 1983); 4hr (Zwadyk, 1986); 4 - 18 hr (Urdea, 1988); 16 - 20 hrs (Chan, 1985). Development times corresponding to those used in these experiments have also been reported: less than 30min (Zeph, 1989) and 10 - 30 min (Haas, 1986). There is no obvious reason why such a discrepancy exists as in several cases the same reagents and similar experimental protocols were used. The effect of over developing blots is to produce a non-specific reaction. While colour intensity increases in all samples it is not proportional and so the real result, sample minus negative control, decreases (Table VIII).

4.1.23 Re-probing of Hybridised Membranes

Radioactive DNA probes once hybridised and detected can be removed from a membrane and it can be hybridised with another probe to detect a different sequence. To determine if it was possible to do this with the streptavidin/alkaline phosphatase detection system a similar method to that used for the removal of a ^{32}P-labelled probe after hybridisation was tried on a biotin-labelled hybridisation (section 3.1.3) While the probe may have been removed the colour precipitate was not, so that it was not possible to reprobe the filter. Nylon filters have been reprobed by first stripping the colour with N,N-dimethylformamide at 65^0C, washing the blot then giving a proteinase K treatment for 1hr at 65^0C. After further washing the probe was removed with a formamide, sodium phosphate treatment for 1hr at 65^0C followed by further

washing. Such a procedure would be justified if the membrane hybridised had taken considerable time, effort or expense to prepare. This procedure is only suitable for nylon membranes (Gebeyuhu, 1987). As the total genomic *Rhizobium* DNA and the subsequent dot or slot blots did not take excessive time, effort or expense to prepare it was decided that since a simple washing procedure failed to remove the colour there was little point in exploring the more complex alternatives. If more than one characteristic was to be examined in a non-radioactive system different probes could be used sequentially with different detection systems that give different colour reactions (Renz, 1984).

4.1.24 Reducing the Cost of the Method

Decreasing the reagent cost was an attractive proposition. Bovine serum albumin was a constituent in three of the buffers used in the detection of hybridisation in the protocol used (section 2.2.15). It functions as a blocking reagent. Heparin (Singh, 1984), Tween-20 (Chan, 1985) and skim milk powder (Reed, 1985; Imaeda, 1988) have been used as blocking reagents in hybridisation procedures successfully. Skim milk powder, being the cheaper option and readily available was chosen as a replacement for bovine serum albumin. Inclusion of 5% filter sterilised skim milk powder in the place of 10% filter sterilised bovine serum albumin in the three buffers in question gave no apparent difference in the intensity of colour produced, the detection limit or the level of background (section 3.1.4) so it was deemed to be an acceptable substitute.

4.1.25 Reducing the System Time to a Minimum

One of the purposes of developing this system was to see if it could be applied as a diagnostic test. Diagnostic tests are often required to be as rapid as possible so the minimum time for this procedure to be carried out was established (10-13hr). The only step where time could be significantly decreased was in the hybridisation which was normally carried out overnight. The minimum hybridisation time was 6-8hr but could be further reduced by the use of 5% dextran sulphate in the hybridisation buffer. With the use of dextran sulphate the absolute minimum hybridisation time was 3hrs, with a total method time of 7-8hr from prehybridisation to detection inclusively (section 3.1.10). This gave a lower colour intensity than longer hybridisations but did

not reduce the limit of detection. This was not as dramatic as the reported reduction of hybridisation time from 72hr to 2hr with a 3-4 fold increase in the signal intensity (Wahl, 1979), however, that result was gained using Southern blots and radioactively labelled probes. Unless the result was required urgently it was more convenient to carry out the procedure over two days with an overnight hybridisation.

4.1.26 Detection Limit

The limit of detection with this system was between 63 and 125 ng. This is much higher than that reported by other workers using the same detection system (Table II) but this may be due to the fact that total genomic DNA was used in this work for both the probe and the slot-blots whereas the higher detection limits were reported using a specific sequence of DNA for the probe and either a dot-blot or a Southern blot as the target. The effect of using a slot-blot as oppossed to a dot-blot or a Southern blot is that the target DNA is spread over a greater area giving a lower concentration of DNA per unit area of membrane. This will effectively reduce the detection limit.

4.1.27 Specificity Compared with ^{32}P-labelled Hybridisations

A comparison between ^{32}P-labelled probes and biotin-labelled probes using this system showed little difference between their specificities. In both cases only other *Rhizobium leguminosarum* species gave a significant reaction to a *Rhizobium leguminosarum* probe (Figure 7 and 8). As it was not possible to distinguish between the biovars of *Rhizobium leguminosarum* with a total genomic probe a specific DNA sequence would be required for this purpose. The inability of total genomic probes to distinguish between closely related species or strains has been reported (Hodgson, 1983; Cooper, 1987; Welcher, 1986).

4.2 CONCLUSION

Non-radioactive probes are becoming more widely used due to their inherent advantages. The system used in this project was originally developed for use as a diagnostic test for bacterial samples obtained from soils and legume root nodules.

Unfortunately crude samples could not be probed directly because the DNA had to be free from other cellular components to avoid the occurence of non-specific reactions. While this system cannot be used to probe crude samples it can be used in place of ^{32}P-labelled probes when pure DNA is used. The detection limit found was higher than that reported by other workers but when using total genomic slot-blots it is feasible to use higher concentrations of target DNA to counteract this. Non-radioactive or radioactive DNA hybridisation with genomic DNA probes can be used to distinguish between species that are not closely related. To distinguish between closely related species or strains a specific DNA sequence is required as a probe since genomic DNA probes show too much cross-reaction for differentiation (Cooper, 1987; Hodgson, 1983; Welcher, 1986).

4.3 ISOLATION OF A SEQUENCE SPECIFIC FOR HOMOLOGY GROUP I OF *BRADYRHIZOBIUM* SP. *(LOTUS)*

4.3.1 Isolation of a Sequence that will Distinguish Between Two Strains of *Bradyrhizobium* sp. *(Lotus)*

Bradyrhizobium sp. *(Lotus)* can be divided into two distinct groups which have approximately 50% homology with each other (Chua, 1984). To distinguish between strain cc814S (homology group I) and strain NZP2076 (homology group II) a DNA sequence specific for strain cc814S was required. Eight clones containing sequences found in strain cc814S but not in strain NZP2076 were isolated from 1 000 colonies (section 4.1.3). As there is approximately 50% homology between strain cc814S and strain NZP2076 theoretically 50% of the colonies representing the gene library should have given a positive result. A possible reason for fewer colonies than expected reacting is that the specific sequences could be relatively small and distributed throughout the genome so that when the DNA was digested many of the specific sequences were in fragments that also contained non-specific sequences and were subsequently removed by hybridisation. The immobilisation of polynucleotides on the solid support (agarose, dextran, cellulose, nitrocellulose) used to retain hybridised sequences has also been suggested as a reason for the low hybridisation efficiencies often associated with these methods (Welcher, 1986). However, the result of the second set of washes taken from the hydroxylapatite in the second experiment showed that 50% of the DNA was removed in single stranded form (section 4.1.4).

When screening for a specific sequence between two species which had 30% homology with each other, 15% of the colonies were picked for secondary screening (Picken, 1987). With 80% homology between two species, 5% of the colonies screened gave a positive reaction (Welcher, 1986). A possible explanation for the low number of positive colonies is that only sequences that are repeated in the genome are hybridising with enough probe to give a recognisable signal. If only repeated sequences are detected by this method, this is an advantage in that they would give a more intense signal when used as probes.

The probe DNA used in the secondary screening was a relatively crude preparation which consequently labeled poorly but as it was hybridised to a large excess of total genomic DNA it produced a satisfactory result although there was a faint reaction with the negative control. To compensate for this the autoradiographs were exposed for a shorter length of time so that no reaction occured between the negative control and the film emulsion. None of the clones showed any distinction between the two homology groups. A similar method used to isolate a specific sequence to distinguish between *Lactobacillus curvatus* and *Lactobacillus sake*, which have 40 - 50% homology, resulted in the isolation of a sequence that distinguished between the test strains and a few other strains but not between all of the strains of *L.curvatus* and *L.sake* (Petrick, 1988). This indicates that greater selection is required to obtain sequences that will distinguish between groups of closely related bacteria rather than between individual strains of bacteria.

4.3.2 Isolation of a Sequence that will Differentiate Between Homology Groups

Isolated specific sequences have previously been used to distinguish between closely related species but not between groups of strains within species. The easiest method for obtaining a sequence specific for a group of closely related organisms is to use a gene known to be unique to that group (Rubin, 1985; Willshaw, 1987; Holben, 1988). This was not applicable to the two groups of *Bradyrhizobium* sp. *(Lotus)* (distinguished by DNA homology) since there are no known homology group specific genes. Greater emphasis was placed on selection for group-specific sequences in the isolation of clones from the gene library. To do this another strain from homology group I was used in place of strain cc814S. This meant that any clones isolated from the gene library would be specific for two strains of homology group I thus increasing the chances of finding a sequence that was common to homology group I while not

found in homology group II. While all of the isolated clones hybridised to varying degrees with the test clones JG207 and JG210 appeared to show a more intense reaction with homology group I strains than homology group II strains (figure 12 A and B). Another clone, JG209, showed an intense reaction with two of the homology group I strains but only a weak reaction with the other homology group I strain (Figure 12C) which excluded it as a possible source of a group specific probe. The variable specificity to other strains and species was also found by Picken (1987) who isolated clones, on secondary screening, that distinguished between the two species being differentiated but reacted with 2 to 4 other species as well.

It was hypothesised that the difference in the intensity of the reaction to the two homology groups by isolates JG207 and JG210 could mean that the segments they carried contained sequences that were common to both homology groups and sequences that were specific for homology group I. Subcloning the sequence could therefore result in the identification of a shorter more specific sequence for use as a probe.This procedure has been reported to be successful (Picken, 1987; Garfinkel, 1989). To determine if this was true in this case one of the isolates was digested into fragments 0.4 to 0.6 Kb long and these were cloned into pGEM2. Specific sequences reported in the literature vary greatly in size (Table XIV). This indicates that the size of the sequence required depends on the application. The insert in the JG210 cosmid was approximately 20Kb long so it was considered that sub-cloning fragments that were approximately 1/40th of this size would give a good chance of separating a specific sequence from the non-specific sequence in the insert.

pGEM2 was used as the vector because it religated and transformed *E.coli* HB101 cells easily. However, pGEM2 does not contain a cloning site in an expressed gene giving a detectable gene inactivated when an insert is ligated into it so it was necessary to decrease the chance of the vector religating to itself by treating the cut vector with calf alkaline phosphatase. The transformants were screened with an insert-DNA probe to detect the colonies carrying the subcloned isolate. When the cosmid library was hybridised with a genomic probe from a representative of homology group I and a representative of homology group II the reactions were not obviously positive or negative. While the intensity of reaction varied from colony to colony nothing could be taken to be a conclusively negative result as most of the colonies showed some level of reaction indicating that non-specific hybridisation had occurred. Since the whole cosmid from the cc814S gene library was subcloned only half of the colonies (containing cc814S sequence as opposed to pLAFR1 sequence)

TABLE XV : Reported Sizes of Specific DNA Sequences used as DNA Probes

ORIGIN OF SEQUENCE	SEQUENCE SIZE	REFERENCE
Specific Gene	0.145 kb x 15 in 14.3 kb	Garfinkel, 1989
	0.85 kb	Willshaw, 1987
	1.1 kb	Holben, 1988
	8.6 kb	Rubin, 1985
Gene Library	0.2 - 1.5 kb	Welcher, 1986
	6.1 kb	Korolick, 1988
Oligonucleotide	0.015 - 0.04 kb	Chollet, 1985
	0.02 - 0.03 kb	Pütz, 1990
	0.02 - 0.03 kb	Hill, 1985
Subcloned Sequences	1.05 - 4.6 kb	Picken, 1987

should have reacted. To isolate a homology group specific sequence it was necessary to be able to distinguish negative reactions from positive reactions. This may have been achieved with a more stringent hybridisation and washing procedure but due to the presence of background non-specific hybridisation in every instance where this method of colony lifts was used, it is more probable that a better result would be obtained with a colony lift procedure that removed more of the cell constituents. The method used by Haas and Fleming (1986) (Table XIV) to remove cellular constituents for hybridisation with a biotin-labelled probe might be suitable. Another control required when a colony blot is screened is to hybridise the membrane with a universal probe that will react with all colonies containing sufficient DNA, after the membrane has been hybridised with the selective probe, the results recorded and the probe removed. This would indicate whether any false negatives had occurred.

Specific probes have been obtained obtained from known specific genes (Rubin, 1985; Holben, 1988; Willshaw, 1987). This is not an option where there are no apparent physiological difference between groups or where the genes coding for physiological differences are unknown. A method for isolating random specific sequences involves cloning random DNA fragments from one species and screening them with genomic probes from the species it is to be distinguished from (Saylers, 1983; Kurtiza 1985, 1986). Where there is low homology between the species to be distinguished between (14 to 28%) this is an appropriate method since a gene library is not required. The screening of a gene library with DNA from several species and choosing colonies that react with the desired strains has been used to isolate specific sequences (Picken, 1987; Korolick, 1988). Selection for specific sequences can be increased by using a group of unique restriction bands (Garfinkel, 1989) or DNA that has undergone a subtraction hybridisation to remove non-specific sequences (Welcher, 1986) to probe a gene library.

4.4 CONCLUSION

Since there was no known physiological difference between the two homology groups of *Bradyrhizobium* sp. *(Lotus)*, a specific gene probe could not be used to distinguish between the groups. The homology level between the two groups was too high to use randomly cloned fragments so selection of a clone from a gene library was required. Subtraction hybridisation to increase the selection for a specific

sequence was considered to improve the chances of isolating a clone carrying a specific sequence. DNA from a homology group I strain that had undergone a subtraction hybridisation with DNA from a homology group II strain was used as a probe to a gene library of an homology group I strain to isolate clones containing *Bradyrhizobium* sp. *(Lotus)* strain specific sequences. Using this method sequences that distinguished between two strains of *Bradyrhizobium* sp. *(Lotus)* were obtained but without further investigation it is not possible to say whether this method could be used to obtain a homology group specific sequence. The method could be improved by placing a greater selection for a group specific sequence on the subtraction hybridisation step. A different homology group I strain was substituted for the one the gene library was made from in the subtraction hybridisation which enhanced the selection of group specific sequences. Further improvement could be obtained if DNA from more homology group II strains was included in the 150ug of unlabelled DNA used as a subtracter, thus removing more common sequences. The use of multiple subtractor strains has been used to obtain specific sequences that could distinguish between *Rhizobium loti* strains effectively (Bjourson, 1988). Multiple cycles of hydroxylapatite separation may also improve the specificity of the method as 5 chromatographic cycles were required before only specific DNA was left in the isolation of sequences specific for *Neisseria gonorrhoeae* (Welcher, 1986).

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