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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

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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.