CHAPTER 1.

INTRODUCTION.

1.1. Context of Thesis Project.

The work for this thesis was carried out at the Institute of Biotechnology at the University of New England, and funded by the Meat Research Corporation. The Institute was working towards two goals in rumen biotechnology. The first, funded by the Meat Research Corporation was to improve rumen fibre digestion by the insertion of bacterial cellulase genes into non-cellulolytic bacteria. The second, funded jointly by the Meat Research Corporation and Applied Biotechnology (Queensland) was to introduce a dehalogenase gene from the soil bacterium *Moraxella species* into a rumen bacterium, to detoxify fluoroacetate, a naturally occurring plant poison responsible for a large number of stock deaths in Queensland and the Northern Territory. In these States, fluoroacetate poisoning is particularly due to the consumption of heartleaf, *Gastrolobium grandiflorum*, and gidyea, *Acacia georginae*.

My particular task within this project was to develop a tracking system for rumen bacteria, particularly those that had been genetically modified. Such techniques, although developed for rumen bacteria, were expected to have general application to the whole field of microbial ecology.

1.2. Ruminants and Rumen Bacteria.

Domesticated ruminants, cattle, sheep, goats, buffalo, reindeer and camels, provide meat, milk, leather and fibre, as well as being important draught and pack animals, and in the case of camels, riding animals. They are a group of animals of major economic and sociological importance throughout the world. And these grazing and browsing animals rely totally on the anaerobic micro-organisms in their rumens to break down the complex carbohydrates, cellulose, hemicellulose and lignin that comprise a large part of their natural fodder. These carbohydrates are anaerobically fermented to short chain volatile fatty acids, which are assimilated through the ruminant intestine. The oxidative

metabolism of these compounds is the major energy source for these animals. Digestion of the rumen microflora provides ruminants with the majority of their protein requirements.

The rumen is a completely anaerobic environment, and its microflora consists of bacteria, fungi and protozoa, of which the bacteria form by far the most numerous and diverse group. Many of the anaerobic rumen bacteria, most of the fungi, and a few of the protozoa, have cellulolytic or other fibrolytic capacity, although the majority of the protozoa are predatory on the bacterial population. The rest of the rumen microfloral population depends on the fibrolytic organisms to produce the short chain polysaccharides they require as a carbon source (Stewart and Bryant, 1988). The rumen microflora make up a complex dynamic ecosystem, whose individual member populations are able to respond to differing food intakes and nutrient levels.

Within the rumen, there are two major environments of importance for rumen bacteria: the liquid rumen fluid and solid food fragments. In order to effect fibrolysis, fibrolytic bacteria must attach themselves to the plant fragments. However, there must also be a proportion of the population of these organisms free in the rumen fluid, moving on and off the plant material. Non-fibrolytic bacteria, dependent on soluble nutrients, associate more with the liquid phase, or are loosely attached to feed particles, or may attach themselves to the rumen epithelium. (Stewart and Bryant, 1988; Olubobokum and Craig, 1990; Cecava *et al*, 1990; Hoover and Miller, 1991).

1.3. Classification of Rumen Bacteria.

Classical taxonomic methods identified about fifteen species of bacteria as making up the major part of the rumen bacterial biomass (Hespell, 1989). However, later studies, notably those of Hudman and Gregg, 1989; Flint and Bisset, 1991; Gregg and Ware, 1990; Mannarelli, 1988; Mannarelli *et al*, 1991, proved that many of the classical "species" of rumen bacteria actually consisted of a large number of genotypically distinct organisms, many of which were genetically unrelated. Thus it became apparent that the rumen was a far more complex system, with a much larger and more diverse population than had been previously supposed.

1.4. Rumen Biotechnology.

With the advent of recombinant DNA technology, it became apparent that it should be possible to manipulate the genomes of organisms, by the introduction of foreign genes, to provide the organisms with new and useful capabilities. The genetic engineering of a multicellular animal is far more difficult and complex than the engineering of a unicellular organism. However, with ruminants, the opportunity exists to influence the metabolism of the whole animal, particularly its feed utilisation, by manipulating the genetic make-up of the unicellular organisms of the rumen. Moreover, because the rumen has such a large and diverse bacterial population, it may be possible to introduce a multiplicity of new genes by the genetic manipulation of a number of species of bacteria. The possibilities for rumen biotechnology have been extensively reviewed (Smith & Hespell, 1983; Teather, 1985; Forsberg *et al,* 1986; Gregg *et al,* 1987; Orpin *et al,* 1988). Suggested goals are summarised in the Table 1.1 below.

Table 1.1.

Suggested Improvements to Rumen Function by Genetic Modification of Bacteria.

IMPROVEMENT	EXAMPLES
Increased production of limiting nutrients	Vitamins , amino acids
Altered proportion of fermentation products	Decreased methane or lactate
Altered rate and extent of degradation of feed products.	Increased fibrolysis, Decreased proteolysis
Production of useful compounds	Anti protozoal compounds Antiparasitic agents Hormones
Removal of unwanted compounds	Plant toxins. pesticides

1.5. Methods Used to Track Specific Strains of Rumen Bacteria.

Previously used methods of tracking rumen bacteria are summarised as follows.

Attwood *et al* (1988) used an RNA probe from a cloned piece of *Bacteroides ruminicola ssp. brevis* B_14 genomic DNA from a plasmid library, selected to be specific for B_14 by DNA hybridisation against several rumen strains. This was used to follow population numbers of B_14 introduced into the rumen. Extracted rumen DNA was spotted onto membranes and hybridised with the P^{32} labeled probe. Filters were autoradiographed, and radioactivity counted.

Stahl *et al* (1987) produced RNA oligonucleotide probes labeled with P^{32} which hybridised to 16S rDNA specific for *Lachnospira multiparus* and probes each specific for two strains of *Bacteroides succinogenes*, S85 and A3, NR9 and DR7, as well as a *B. succinogenes* "signature probe". These were used to track levels of these strains in the rumen after monensin treatment. Total rumen nucleic acids were extracted by mechanical means and loaded onto filters. Oligonucleotide probes were hybridised to the target nucleotides, and visualised by autoradiography.

Flint *et al* (1989) produced rifampicin resistant mutants of *Bacteroides multiacidus* and *Selenomonas ruminantium*, and followed their fate after re-introduction into the rumen by selective plating.

Brooker and Stokes (1990) used monoclonal antibodies and fluorescent labeling or ELISA against strains of *Selenomonas ruminantium*. (See 1.7. for a fuller description).

Gregg (1991, pers. comm., Gregg *et al*, 1993) used a combination of selective culture and colony hybridisation to follow population levels of *Butyrivibrio fibrisolvens* E14 introduced into sheep rumen. E14 had been genetically modified to express tetracycline resistance by conjugative transfer of the *Enterococcus faecalis* transposon Tn916. Rumen samples were plated onto tetracycline media and resultant colonies probed at very high stringency with P^{32} labeled E14 chromosomal DNA. Results were visualised by autoradiography.

None of these methods seemed to fully satisfy all the requirements for a flexible and sensitive tracking system for rumen bacteria.

1.6. Tracking Systems for Rumen Bacteria: Specific Requirements.

Several requirements had to be taken into account when deciding the best method to be used, the first requirement being that the method be able to cope with the complexity of the bacterial populations of the rumen.

It is probable that genetic modifications will be developed, at least initially, for single genetic strains of bacteria, as gene expression systems and vectors tend to be specific for individual genotypes. Therefore any tracking system must be able to distinguish bacteria on the basis of genotype, rather than the phenotypic characteristics of classical taxonomic methods. It is also desirable that the tracking method be adaptable to track unaltered as well as genetically modified organisms (GMOs), so as to be able to determine distributions and population levels of parent strains of potential GMOs. Further, the system should be useable for many strains of bacteria, and have the ability to distinguish between them.

Another requirement was the need for a highly sensitive technique for the detection of very low levels of particular bacterial strains. The population of any particular strain of bacteria in the rumen has been shown to fluctuate markedly over time (Gregg *et al,* 1993; Stahl *et al,* 1988), even though the population of any particular phenotypic genus remains relatively constant. Also, when considering the release of a genetically modified organism (GMO) into the rumen, it is important to be able to monitor it, not only in the animal to which it has been introduced, but also in the environment, such as pastures and drinking water. It is important to know whether the bacterium can survive or spread outside its host species. Thus, the ability to detect very small numbers, preferably down to a single copy, of the organism of interest, was a strong requirement. In addition, the method needed to be able to detect the organism of interest against a heavy background of non-target organisms, not only in rumen samples, but also in environmental samples such as soil or water, or in faecal samples.

The final requirement was a tracking technique that was capable of giving quantifiable results, so that not only the presence or absence of the organism could be established, but also the level at which it was able to maintain itself in the rumen, whether this level fluctuated or not, and whether its population could be manipulated by external means such as dietary changes. With these criteria in mind, the literature was searched for methods that might be able to fulfill them.

1.7. Molecular Methods for Tracking Bacteria in the Environment.

Molecular methods for tracking bacteria were reviewed by Pickup (1991), and Saint (1993), and detection methods based on DNA hybridisation were reviewed by Sayler and Layton (1990). These reviews cover the known methods of detecting single strains of bacteria in environmental samples. Gregg (1991) briefly discussed methods of detection suitable for rumen bacteria.

Some detection methods rely on pre-culture of the bacteria. These include detection by selective media, of such traits as antibiotic resistance, or marker genes, whether natural or introduced, such as β-galactosidase, or *xyl E*, which produce coloured colonies on specific media. Alternatively, cultured colonies can be screened by colony hybridisation.

Methods based on culturing were deemed unsuitable for the purposes of this project for two main reasons. First, any single bacterial strain is likely to be present as only a small proportion of total rumen bacterial biomass (Gregg, 1991), and selective media do not exist to identify even classical phenotypic "genera". Therefore it would seem necessary, as shown above by Flint *et al* (1989) and Gregg *et al* (1993) to introduce a selective genetic trait into the organism of interest. Second, rumen samples must be cultured under strict anaerobic conditions soon after collection, as they have limited viability, presenting problems with field sampling.

Systems based on introduced markers were also unsuitable to the purposes of the project. First, only genetically altered stains can be monitored by such methods, not their parent strains. Second, unless a multiplicity of marker genes was used, one for each GMO, it would be difficult to monitor more than one GMO in the rumen. Moreover, gene transfer methods would have to be developed for marker genes in each GMO.

Other methods based on direct detection of bacteria without culture can be based either on immunological methods or on some form of nucleic acid hybridisation.

Immunological methods depend on the manufacture of antibodies that bind specifically to the genetically modified organism. The antiserum, or monoclonal antibody (mAb), may be produced against a native antigen, or against an introduced marker gene. Morgan *et al* (1989), developed an enzyme-linked immunoabsorbent assay (ELISA) against the enzyme catechol 2,3-dioxygenase expressed by the *Xyl E* gene transformed into *Pseudomonas putida*. However, in order to detect the protein, it was necessary to

lyse the cells, and considerable trouble was reported with background staining. Once these problems were overcome by pre-treatment of the titre plates, he was able to report detection levels down to 10^3 cfu/ml of lakewater. It is worth noting however, that the *Xyl E* gene was constitutively over-expressed in his transformants, so that unusually large amounts of protein were available for detection.

However, most antibody preparations are raised against whole cells, and it is not possible in these cases to identify the specific antigen. Both cell surface proteins, including flagellar proteins, and capsular polysaccharides are likely antigenic candidates.

Brooker and Stokes (1990) produced a series of monoclonal antibodies against strains of the rumen bacterium *Selenomonas ruminantium*, and were able to produce species specific and strain specific antibodies to cultured *S. ruminantium.* They were able to show that at least one of their mAbs appeared to be specific for a carbohydrate antigen, while another was reactive with two different sized PAGE separated proteins. However, they were unable to get reactivity against several freshly isolated strains of S. *ruminantium,* and admitted that antigenic activity seemed to be partly dependent on culture conditions. No attempt to test the antibodies against rumen samples was reported.

These results made it doubtful that it would be possible to raise strain specific antibodies, against a range of bacterial strains, which would be completely specific and equally reactive against bacteria under all the differing nutrient conditions that undoubtedly arise in the rumen. Therefore immunological methods were not considered to be the most suitable tracking method.

Genetic detection methods are based on the ability of complementary nucleotide sequences to bind to each other. The target DNA is denatured to single strands, and probed with a single stranded or denatured probe, which is generally labeled in some way to facilitate detection. DNA hybridisation methods for detecting specific organisms in the environment were reviewed by Sayler and Layton (1990).

For DNA hybridisation, DNA sequences bonded to nitrocellulose or nylon membranes may be probed with labeled DNA, RNA or oligonucleotide specific to the organism of interest, or to an inserted marker gene, and this technique has been widely used for detecting genetically modified bacteria. (Sayler & Layton, 1990; Pickup, 1991; Saint, 1993). The probe may be labeled with a radio-nucleotide such as ^{32}P ATP or non-isotopically labeled with a variety of chromogenic or fluorogenic labels. The target DNA

may be derived from colonies lysed on a filter (colony hybridisation), or extracted from the sample to be tested and loaded onto a filter (dot blot/slot blot hybridisation). Both these techniques are well known, and have been very widely used.

Radiolabeled RNA probes have been hybridised to total rumen DNA on filters (Attwood *et al*, 1988), to detect specific rumen bacterial strains (see section 1.6. above). RNA probes, being single stranded, are more sensitive than DNA probes, but are more difficult to produce and require extra care in handling.

An alternative approach, claimed to be more sensitive than filter hybridisation, was used by Steffan & Atlas (1990), to detect genetically modified *Pseudomonads* released into the environment. A plasmid specific RNA probe was hybridised to extracted target DNA in solution. The DNA-RNA hybrid was then isolated on a Sephadex column, and detected by liquid scintillation counting.

Oligonucleotide probes complementary to strain specific 16S rRNA sequences have been used for the detection of specific ruminal bacterial strains, in combination with hybridisation against total rumen nucleotides (Stahl *et al,* 1988, see section 1.6. above). 16S rRNA probes can be made extremely strain specific by taking them from the variable regions of the sequence, or species specific, by using more conserved regions. They also have the advantage that there are large numbers of bacterial ribosomes, the number varying with species, and with the growth rate of the organism. Therefore the probes have enhanced sensitivity over those that target single copy sequences. However, the system was not quantifiable, as there was no way to determine how much of the extracted nucleotide was of plant origin.

Amman *et al* (1992) used strain specific, fluorescently labeled, 16S rRNA oligonucleotide probes for detecting specific bacteria in culture. Detection of the fluorescently labeled organism was by flow cytometry. Bacterial cell walls were permeabilised, and the probes targeted to the bacterial ribosomes. As *E. coli* contains 10^4 - 10^5 ribosomes per cell (Amman *et al*, 1992), and similar numbers can be assumed for other bacterial species, the ribosome provides a naturally amplified target.

While the nucleic acid hybridisation techniques outlined above undoubtedly have, or can be made to have, the required specificity to satisfy the tracking system criteria discussed in Section 1.7, they unfortunately lack the required sensitivity. Table 1.2, after

Pickup (1991), shows the sensitivity achieved by the various tracking techniques discussed.

Table 1.2.

Sensitivity of Bacterial Detection Systems.

Method	Sensitivity Cells/ml	Background Cells/ml	Reference
ELISA	10^3	10^6	Morgan *et al*, 1989
ELISA	10^3	N.D.	Brooker & Stokes, 1990
DNA Hybridisation	10^7	$\sim 10^{10}$	Attwood *et al* 1988
DNA Hybridisation	10^3	10^6	Morgan *et al*, 1989
Selective plating	10^3	10^{10}	Flint *et al*, 1989
Selective plating & Colony Hybridisation	10^4	$\sim 10^{10}$	Gregg *et al*, 1993
16S rRNA Hybridisation	10^5	$\sim 10^8$	Amman *et al*, 1990

Lack of sensitivity, identified as a major problem with the methods surveyed above, was in large part due to the fact that the target organism was present as a very small proportion of the total population.

When the proportion of target could be selectively amplified with respect to its background, as in selective plating followed by colony hybridisation (Gregg et al, 1993), sensitivity was improved.

The polymerase chain reaction (PCR) is a method of selectively amplifying a defined target sequence, able to amplify very small numbers of target sequence to very high copy numbers. This method had been used to detect specific bacteria from environmental samples (see chapter 1 section 12).

1.8. The Polymerase Chain Reaction.

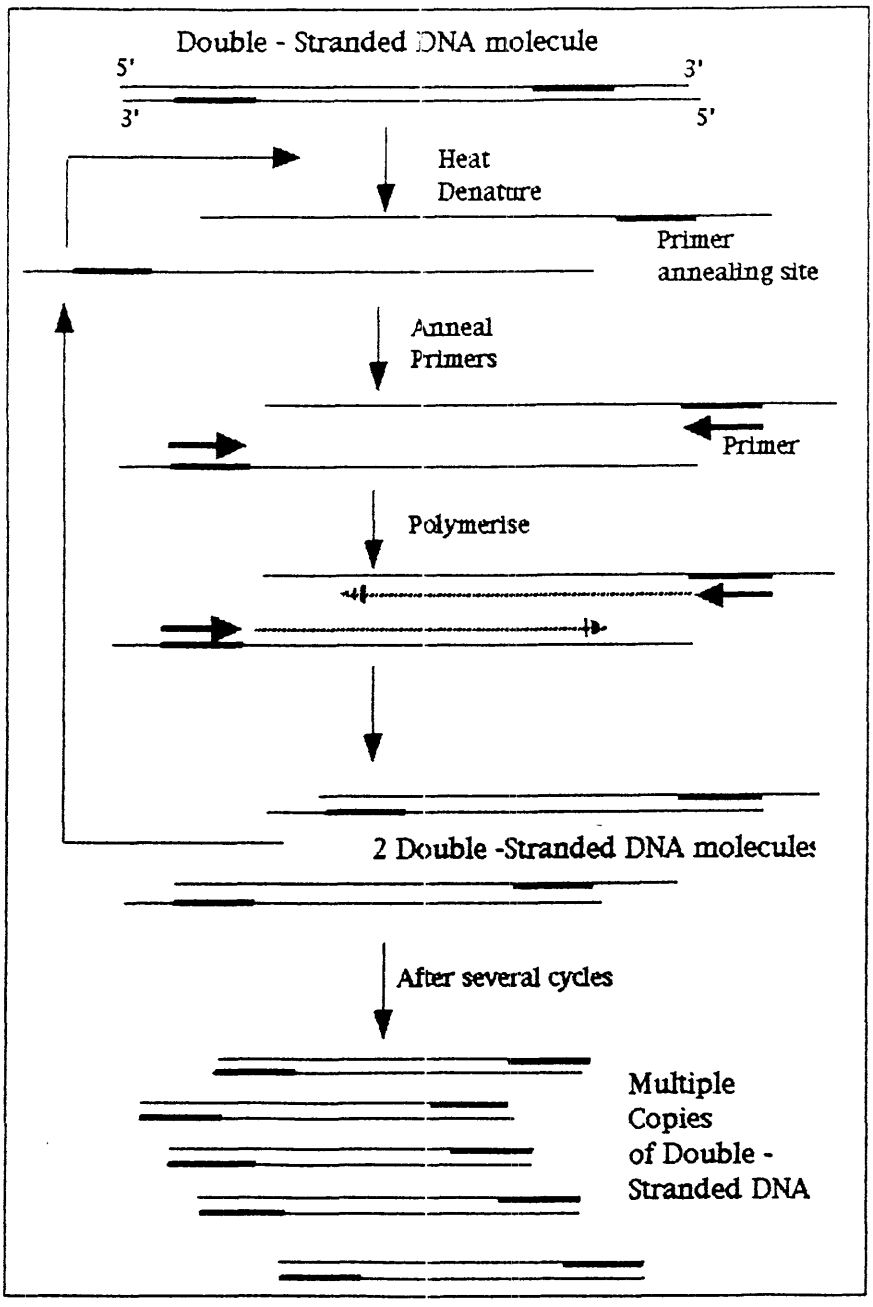
The Polymerase Chain Reaction (PCR) is an *in vitro* method of DNA amplification, in which denatured DNA undergoes simultaneous amplification on both strands (Mullis and Falloona, 1987). The sequence for amplification is defined by a pair of oligonucleotide primers, which anneal to specific sites on heat denatured DNA, one primer to the sense strand of the DNA, and the other to the antisense strand, with the annealing sites selected so the primers direct synthesis towards each other. Thus, after the first polymerisation step, there are two copies of the original sequence. After each round of synthesis is completed, the DNA is re-denatured, and the process repeated. (See figure 1.1). Thus the process provides exponential amplification of the target DNA source.

In the original protocol (Mullis and Falloona, 1987) the enzyme used was the Klenow fragment of *E. coli* DNA polymerase I. This enzyme is destroyed by the high temperatures necessary to denature DNA, so it was necessary to add enzyme at each cycle. The use of thermostable DNA polymerase from the thermophilic bacterium *Thermus aquaticus* (Taq polymerase) (Saiki *et al,* 1988), overcame this problem, and made automated cycling possible. The reaction is normally cycled among three temperatures: a high temperature step for DNA denaturation; a lower temperature to allow the primers to anneal; and an intermediate temperature for polymerisation.

The reaction takes place in a buffered solution. The normal buffering agent is Tris at pH 8.3-8.8. Essential reaction components are oligonucleotide primers, deoxynucleotides, magnesium ions, and Taq DNA polymerase. Potassium chloride may be present to enhance annealing; detergents and proteins such as gelatine may be included to stabilise the enzyme

Figure 1.1.

THE POLYMERASE CHAIN REACTION.



Thickened lines represent primer binding sites, and heavy arrows represent oligonucleotide primers, with arrows showing direction of polymerisation, 5' to 3'. Initial amplification products are of random lengths, but as the amplification products act as templates, specific amplification products, whose length is limited to the primer - primer sequence, become predominant.

1.9. Control of PCR Specificity and Sensitivity.

The specificity and sensitivity of PCR depend on particular reaction parameters. The specificity of the reaction depends largely on the choice of the oligonucleotide primers, and the temperature at which they are annealed (Wu *et al,* 1991; Kwok *et al,* 1990; Sommer and Tautz, 1989). The longer the primer, the less is the probability of its having more than one target site on the genome (the probability of occurrence of any sequence is given by 4^n, where n is the number of nucleotides in the sequence). In practice, primers of 20- 30 bases are commonly used. The temperature at which the primer anneals should be selected to be near the melting point of the primer-oligonucleotide duplex. Wu *et al* (199), provided a method for calculating maximum annealing temperature. If lower temperatures of annealing are used, there is an increased possibility that the primer may anneal to a less than perfectly complementary site. Indeed this possibility is much exploited in the use of the PCR reaction for mutagenesis by the deliberate annealing of mismatched primers. If very long primers are used, the optimum annealing temperature rises above the optimum polymerisation temperature (approx. 72°C), and either stringency of annealing must be sacrificed, or annealing temperature raised to a point at which the enzyme function is compromised (Wu *et al,* 1991).

Enzyme concentration can also have an effect on the specificity of the reaction. Too high an enzyme concentration can lead to the accumulation of non-specific amplification products. Where there is a problem with non-specific amplification, agents which increase specificity, such as formamide (Sarkar *et al,* 1992) or tetramethyl ammonium chloride (Hung *et al,* 1990), can be added to the reaction mix. Chou *et al.* (1990) reported improved specificity by using a layer of wax to separate template and enzyme from other reaction components until the reaction tube has reached a high enough temperature to melt the wax, and allow components to mingle.

The sensitivity of the PCR reaction is governed by the number of cycles through which the reaction is taken. In theory, each cycle of the reaction doubles the yield of product. In practice, the efficiency of the reaction varies. Primer choice can have a very considerable impact on the efficiency of the reaction, though the reasons for this are not fully understood (Innes, 1990). When primers contain regions of internal complementarity, or primer pairs are mutually complementary, efficiency of target annealing will of course be markedly reduced. However, when these problems are absent, it may still not be

possible to achieve an efficient reaction. Adjustment of the concentrations of the reaction components, especially the magnesium ion concentration can have very significant effects on reaction efficiency. Again, the reasons are not known, though it seems plausible that at least part of the answer may lie in the secondary structure of the target DNA.

1.10. Other PCR Parameters.

The time for which the reaction needs to be denatured will depend on the length of the template DNA. Melting time is well known to be proportional to the length of the DNA molecule, and the number of G and C residues. When using genomic DNA as template, the first one to five cycles are given a longer denaturing time, of two to five minutes. After the first few cycles, the concentration of shorter amplification product molecules will greatly outstrip that of the template, and denaturing time is then reduced for the remaining cycles. This reduction in the time spent at high temperature has the dual benefit of reducing the time for the whole reaction sequence, and increasing the number of cycles for which the enzyme remains viable.

The time of extension or polymerisation is governed by the length of the product DNA, which is specified by the distance between the primers on the target DNA. As a rough guide, an extension time of 1 minute per kilobase of DNA has been suggested (Innes, 1990).

The ramp time, or time for the temperature of the reaction mixture to change from one temperature to the next varies according to the volume of the reaction, the thickness of the reaction tube, and the design of the thermocycler. Reactions are normally carried out in volumes of 10-100 µl, in microcentrifuge tubes. Thermocyclers come in essentially two designs: either the tubes are held stationary in a metal block, whose temperature is re-adjusted to each stage of the reaction; or the tubes are moved between water baths, each of which can be set to the correct temperature for each stage of the reaction. The block cyclers have the advantage of compactness, but have longer ramp times than water bath thermocyclers. Also block thermocyclers tend to have some degree of temperature variation across the block, whereas waterbaths can be kept uniform in temperature across the waterbath, by using a large volume of water, and keeping it stirred. While the effect of

ramp time on the PCR reaction is not known, temperature variations between tubes can make a difference to the reaction yield.

1.11. Control of Contamination in PCR.

Because PCR is so sensitive, very small traces of target DNA can be readily detected. This means that very great care must be taken to avoid introducing accidental contamination to PCR reagents.

There are two approaches to the problem of excluding contaminants from PCR. The first is to use extreme care in sample and reagent preparation, and set up working conditions so as to minimise the chance of accidental carry over of amplified products or target DNA (Kwok and Higuchi, 1989). This involves setting aside special areas for PCR preparations and DNA preparation, providing separate pipettes for handling PCR reagents, using positive displacement pipettes to avoid DNA contamination of pipette barrels, and ensuring that tubes containing amplification products amplification products are never opened anywhere near PCR preparation areas. Preparation areas can be sterilised by bactericidal UV lighting. Non-degradable reagents can be sterilised by autoclaving. As a precaution, reagents are aliquotted into small volumes, so that if contamination does appear, all reagents in use can be disposed of readily. Gloves should be worn at all times and should be changed frequently. All PCRs should be run with negative controls to check that contamination is not present.

The second approach is to accept that periodic contamination may well be inevitable, and attempt to deal with the problem rather than the cause. Cimino *et al* (1991) suggest that the majority of contamination arises from the spread of amplification product molecules which are present in very high numbers, up to 10^{12}/100 ml (Kwok and Higuchi, 1989). Once these are handled in any way they have a high potential for dispersing through the laboratory as aerosols. Therefore Kwok and Higuchi developed an in-tube post amplification sterilisation technique. Psoralens were included in the reaction, and after amplification, were photo-activated to form compounds that damaged the DNA, so as to prevent it acting as a PCR template. This procedure is obviously unusable if the DNA is required for sequencing or cloning.

DeFellipes (1991) and Zhu *et al* (1991) used enzymic pre-treatment of PCR reaction components to destroy any contaminating target material that might be present. Zhu *et al* used exonuclease III, which is specific for double stranded DNA, to destroy contaminants, while DeFellipes used restriction enzyme treatment. Both treatments were used prior to the addition of the template DNA, to damage accidentally incorporated target molecules so they could no longer be amplified.

1.12. Detection Methods Utilising the Polymerase Chain Reaction.

It was known that the polymerase chain reaction was a highly sensitive detection procedure in the presence of large quantities of non-target DNA. Saiki *et al* (1988) had reported detection of a single copy of a specific β-globulin gene in 1 mg of non-target human chromosomal DNA.

The technique had been used for detection of a number of specific bacteria, both genetically modified and unaltered, in environmental samples.

Steffan and Atlas (1988) used PCR to detect the herbicide-degrading bacterium, *Pseudomonas cepacia* AC1100, after it had been added to samples of river sediments. DNA was extracted from the samples and purified by caesium chloride density gradient centrifugation. The target was a 1 Kb section of a 1.3 Kb repeat sequence present at 15 to 20 copies on the *P. cepacia* genome. Primers were 24mer consensus primers, as the repeat sequences were not identical. This necessitated a low annealing temperature, 40°C. However, the primers proved specific for the target organism. Thirty cycles of amplification were used, and the resultant reaction mixture was loaded on to filters and probed by dot blot hybridisation. A sensitivity of one *P. cepacia* cell per gram of sediment was reported.

Chaudry *et al* (1989) used genetically modified *E. coli* transformed with a plasmid containing, as target sequence, a 0.3 Kb insert of Napier grass DNA. Bacteria were inoculated into filter sterilised sewage and lakewater. They reported that 20 cycles of PCR amplification, coupled with dot blot hybridisation procedures, enabled them to detect the GMOs in these samples for at least 4 days longer than by selective plating methods. By implication, the PCR/DNA hybridisation method was considerably more sensitive.

They estimated that this method would detect 1000 copies of their target sequence, and that sensitivity was not affected by the presence of 1 mg non-target DNA, or 1 mg tRNA. They also suggestedythat sensitivity would be improved with more PCR cycles.

Bej *et al* (1990) developed PCR probes for *E. coli* from sequences on the *lacZ* and *lamB* genes. They reported that by varying the annealing stringencies, the *lamB* probe could be used to detect a variety of enteric bacteria at low annealing stringencies, but was specific for *E. coli, Salmonella typhimurium,* and all *Shigella* species tested, at high annealing stringencies. The *LacZ* probe was also non-specific at low annealing temperatures, but specific for *E. coli* and *Shigella* species at higher annealing temperatures. These results show the importance of using exactly matched primers and stringent annealing conditions when trying to distinguish closely related organisms. Sensitivity reported was 1 cell/100 ml water. Bej *et al* tested several detection methods for the PCR products. These were, in increasing order of sensitivity, electrophoresis on agarose or acrylamide gels stained with ethidium bromide, DNA hybridisation to DNA transferred from gels to filters by Southern blotting, and dot blot hybridisation.

Joshi *et al* (1991) used gene specific primers to amplify regions of the histidine permease operon in *S. typhimurium* and *E. coli.* They showed that it was possible to use intact cells as PCR template, instead of extracted DNA, even when the target sequence was encoded by chromosomal rather than plasmid DNA. They reported that the use of intact cells resulted in no loss of sensitivity. It is noteworthy that Joshi *et al* did not report using a PCR buffer containing detergent, and did not find it necessary to extend the initial denaturation step beyond 1 minute. Visualisation of amplification product was on ethidium stained agarose gels.

Other reported detection systems using PCR include enterotoxic *E. coli* (Olive, 1989); *Legionella pneumophila* (Starnbach *et al,* 1989); *Shigella flexneri* (Lampel *et al,* 1990); *Chlamydia trachomatis* (Welch *et al,* 1990); but these add little to the methods discussed above.

From the reports surveyed, it appeared that PCR amplification of strain specific gene sequences satisfied the criteria for a tracking system discussed above. It was highly sensitive, even in the presence of a very high non-target DNA background. It could be made completely specific by using primer pairs complementary to unique sequences on the target genome, together with high annealing stringencies. It could detect any target

organism for which some DNA sequence was known, and could detect recombinants by designing probes specific for the imported genetic material. The only criterion which had not been demonstrated was quantitation.

1.13. PCR Quantitation.

The PCR reaction is difficult to quantify, because the increase in product is exponential, so minute differences in reaction conditions can make a large difference in the final amount of product, making it difficult to relate the amount of product to the amount of template DNA. However quantitation has been achieved by a number of workers.

The standard method for quantitation of PCR reactions is the competitive PCR technique developed by Gilliland *et al* (1990). They hypothesised that if two DNA templates were co-amplified in the same reaction tubes, then the differences in reaction conditions would apply to each set of templates equally. Therefore, the ratio between the two products would be unaffected by these differences, and could be used to quantitate the reaction. They suggested using two templates, which differed only by the presence or absence of a restriction site, or two templates, which differed in length. For the latter, a cDNA, and its genomic sequence, which included a small intron was suggested. In both cases, the same set of primers could be used to amplify both templates.

However, other researchers have quantitated the PCR reaction using competitive amplification of internal standards, and comparing the amount of product from each template, but without plotting product ratios. Chelly *et al* (1988) used this method to quantitate transcription of the human dystrophin gene, using the housekeeping gene Aldolase A as the internal standard. Reverse transcription of the mRNAs preceded the PCR amplification. Wang and Mark (1990) used a synthetic DNA plasmid insert, which comprised the primer pairs for a number of specific target genes, to generate a cRNA which could be used as an internal standard for a number of gene products. Kellogg *et al* (1990) used a portion of the histocompatibility gene HLA, as an internal standard to quantitate HIV-1 proviral DNA, but found difficulties with differing amplification efficiencies of the two templates. These methods appeared to require amplification to be kept within the exponential stage of the PCR reaction in order to yield quantitative results.

Murphy *et al* (1990) quantitated the expression of the *mdr* -1/PGP gene in two different cell lines under different levels of inhibition without using internal standards. The mRNA was converted to cDNA by reverse transcription, and PCR performed on the cDNA using gene specific primers. They found that as long as the reaction was kept within its exponential stage, it was possible to quantify the reaction by comparing the results from serial dilutions of templates, or from different numbers of cycles using the same template. They also found that the inclusion of a co-amplified control sequence lowered the number of cycles, or the number of serial dilutions over which the reaction remained exponential.

Siebert and Larrick (1992) reviewed competitive PCR methods and concluded that the major advantage of using a competitive system which utilised product ratios for quantitation was that it was not necessary to keep within the exponential range of the reaction. They also emphasised the importance of using co-amplificants with similar amplification efficiencies.

While most of the examples of competitive PCR to date have been concerned with mRNA quantitation of gene expression, there seems no reason to doubt that the technique can be extended to the quantitation of any PCR amplified template.

1.14. Summary.

Consideration was given to the best method to use for tracking rumen bacteria *in vivo*. Methods based on identification of species specific gene sequences offered the best chance of distinguishing phenotypically similar but genetically different bacterial strains. The polymerase chain reaction was reported to amplify sequences specific to any given target organism, and promised the sensitivity to detect even one cell against very high background levels of non-target organisms. Of considerable interest was the possibility of using intact cells as template material. PCR quantitation methods, holding promise for counting target organisms in rumen samples, were available. In short, PCR seemed to satisfy all the requirements for a successful rumen bacterial tracking system, and to overcome the main deficiencies of previously used methods.

CHAPTER 2.

MATERIALS AND METHODS.

2.1 GENERAL MATERIALS AND METHODS.

2.1.1. MATERIALS.

(i). General Chemicals.

These were puchased from Sigma, BDH, or Aldrich, were of analytical grade, and were used without further purification, except for phenol and ethanol, which were of laboratory grade, and purified by redistillation.

(ii). Special Chemicals and Kits.

Agarose)	
Sure Clone kit)	Pharmacia.
Dithiothreitol)	
Agarose	Progen.
Qiagen plasmid preparation kit)	
α^{32}P Oligo labelling kit)	Bresatec.
Gene Clean kit)	
$\alpha\ ^{32}$P dATP and $\gamma\ ^{32}$P ATP	Dupont.
Deoxyribonucleotide triphosphates	Boehringer Mannheim.

(iii). Bacteriological Materials were purchased from Difco and Oxoid

(iv). Enzymes.

RNAse I	)	
Lysozyme	)	Boehringer Mannheim.
Proteinase K	)	
Polynucleotide kinase	)	
T4 ligase	)	Pharmacia.
Klenow fragment of DNA polymerase I	)	
DNA polymerase I	)	

(v). Restriction Enzymes.

Bam H I	)	
Eco R I	)	
Hind III	)	Pharmacia.
Rsa I	)	
Sau 3a	)	
Hind III		Progen / Pharmacia.
Stu I		New England Biolabs.

(vi). Buffers.

Note pH readings were at room temperature, ~22°C, unless otherwise specified.

Percentages are in w/v for solids and v/v for liquids

TE Buffer	10 mM Tris HCl
	1 mM EDTA
	Adjusted to pH 8 with conc HCl

TAE Buffer	40 mM Tris base	)
	20 mM Na acetate	) Prepared as
	1 mM EDTA disodium salt	) 20 x concentrate
	pH 8.2	

TBE Buffer	130 mM Tris base	)
	45 mM boric acid	) Prepared as
	2.5 mM EDTA disodium salt	) 10 x concentrate
	pH 8.8	

Restriction Enzyme Buffer (REB)

	20 mM Tris HCl	)
	10 mM $MgCl_2$	) Prepared as
	1 mM dithiothreitol	) 10 x concentrate
	50 µg/ml bovine serum albumin	)
	pH 8.3 @ 37°C	

Ligation Buffer pH 7.5

	60 mM Tris HCl	)
	10 mM $MgCl_2$	) Prepared as
	10 mM dithiothreitol	) 10 x concentrate

100 x Denhardt's Solution

2% polyvinylpyrrolidone

2% ficoll

2% bovine serum albumin

Prehybridisation Fluid

50% formamide

5 x SSC

5 x Denhardt's solution

1% glycine

50 mM K_2HPO_4/KH_2PO_4 pH 6.2-6.8

200 mg/ml herring sperm DNA, sonicated and heat denatured

Hybridisation Fluid

50% formamide

5 x SSC

1 x Denhardt's solution

20 mM K_2HPO_4/KH_2PO_4 pH 6.2-6.8

10% dextran sulphate

100 mg/ml herring sperm DNA, sonicated and heat denatured

Gel Loading Buffer for agarose gels

0.02% bromphenol blue

50% glycerol

5mM Tris HCl

pH 7.5

Ficoll Loading Buffer for acrylamide gels

0.025% bromphenol blue

0.025% xylene cyanol

15% ficoll, in H_2O

Stock Acrylamide Solution

40% acrylamide

1% bis-acrylamide

(vii). Bacterial Media.

All bacterial growth media were sterilised by autoclaving at 107°C for 45 minutes.

LB Broth
1% bacteriological peptone
1% tryptone
0.5% NaCl

SOC Medium
2% bactotryptone
0.5% yeast extract
2.5 mM KCl
10 mM $MgSO_4$
10 mM NaCl
10 mM $MgCl_2$
20 mM glucose, added after sterilisation

Rumen Fluid Medium
33 ml salt solution A
33 ml salt solution B
33 ml clarified rumen fluid
100 ml water
0.2 g peptone
0.2 g yeast extract
1.0 g $NaHCO_3$
0.4 g glucose
0.4 g cellobiose
0.1% resazurin
40 mg cysteine

Salt Solution A
0.3% KH_2PO4
0.6% NaCl
0.3% $(NH4)_2SO_4$
0.03% $CaCl_2$
0.03% $MgSO_4$

Salt Solution B
0.3% K_2HPO_4

Agar plates were made from the appropriate media by the addition of 1.5% agar. Ampicillin, for the selection of transformants, was added to a concentration of 100 µg/ml to both plates and broth.

2.1.2. METHODS

(i). Restriction Enzyme Digests.

These were carried out either in the manufacturer's buffer, or in REB, with salt concentrations adjusted to manufacturer's specifications, and with the addition of 1 mM spermidine. Digests were normally carried out in 100 µl and incubated for 90 min at the specified temperature.

DNA was recovered by adding 0.1 volumes 3M Na Acetate, pH 4.3, and precipitating with 2.5 volumes of ethanol.

(ii). Plasmid DNA Preparations.

Note. All phenol extractions were carried out with phenol buffered to pH 8 with TE buffer.

a) Alkaline Lysis Method.

1.5 ml overnight culture of *E. coli* was centrifuged, and the supernatant discarded. Bacteria were resuspended in 100 µl freshly made lysozyme solution (15% sucrose, 10 mM EDTA, 25 mM Tris HCl pH8, 0.2 mg/ml lysozyme) at room temperature for 5 min. 300 µl 0.2M NaOH/1% SDS, was added, and the tube incubated on ice 5 min. 150 µl ice-cold 3M K Acetate pH 4.8, was added to precipitate protein and genomic DNA, mixed without vortexing, and incubated on ice for 5 min. The precipitate was removed by centrifugation, and the supernatant extracted once with 1:1 phenol/$CHCl_3$, and twice with $CHCl_3$. Plasmid DNA was precipitated from the aqueous layer with 2-2.5 volumes of ethanol at room temp for 5 min. The pellet was washed with 70% ethanol, and dissolved in 20-50 µl water.

b) Modified Heat Shock Method.

100 ml of overnight culture was centrifuged and the pelletted cells washed once in TE buffer. Cells were resuspended in 1.5 ml 15% sucrose, 50 mM Tris HCl pH8, 50 mM EDTA with 0.5 ml freshly prepared lysozyme (8mg/ml), and incubated 15 min at room temp. and 30 min on ice. 1.5 ml ice cold water was added and mixed and the tube incubated on ice 5 min and at 70°C for 20 min. Precipitated DNA and proteins were

removed by centrifugation, and 10 µl RNAse A (10 mg/ml), was added and the mixture incubated at 37°C for 20 min. 10 µl proteinase K (10 mg/ml) was added and incubated at 50-55°C for 30 min. Solvent extraction and DNA precipitation were as for the alkaline lysis method. DNA was dissolved in 0.5 ml water.

c) Qiagen Method

This is a plasmid preparation claimed to give plasmid DNA of a purity comparable to that obtained by caesium gradient ultracentrifugation. Preparations were carried out according to the manufacturer's instructions.

Briefly, cells were alkaline lysed in the presence of RNase A. Denatured genomic DNA and proteins were precipitated with acid potassium acetate and the supernatant loaded onto a resin column. RNA and proteins were eluted at low salt concentrations, and plasmid DNA released by higher salt concentrations.

d) Plasmid Caesium Chloride Density Gradient Centrifugation.

This was carried out according to the method of Sambrook et al. (1989).

Briefly, a preparation of plasmid DNA, was brought to a density of 1.55 g/ml with caesium chloride. The solution also contained ethidium bromide at a concentration of 600 µg/ml, to maintain the plasmid in the supercoiled state. This was centrifuged for 36 hr at 250,000 g. The supercoiled DNA, denser than linear or nicked circular DNA, segregated as a separate band, which was collected and cleaned by dialysis against TE buffer.

(iii). Genomic DNA Preparation.

This method was used for preparation of genomic DNA from laboratory cultures of rumen bacteria , and is a slight modification of the method of C. Ware (1992).

1.5 ml of stationary or late log phase culture was centrifuged to pellet the cells. and the pellet washed twice in TE buffer. Cells were then suspended in 300 µl freshly prepared lysozyme (8 mg/ml in TE buffer), and incubated at 37°C for 30 min. 10 µl each of 10% SDS and RNAse A (10 mg/ml) were added , and the mixture incubated at 37°C for 30 min. 10 µl Proteinase K (10 mg/m) was added, and the mixture incubated for 1-2 hours at 55°C until lysis was complete. Undissolved capsular material was removed by centrifugation, and the supernatant extracted once with 1 vol 1:1 phenol/ $CHCl_3$, and

twice or three times with $CHCl_3$. DNA was precipitated from the aqueous layer with 2.5 volumes ethanol, or 1 vol propan-2-ol at room temperature. DNA was pelletted by centrifugation, the pellet washed in 70% ethanol, and dried under vacuum. DNA was dissolved in 100 µl H_2O.

Note. With *P. ruminicola* AR20, there was sometimes a white precipitate, with ethanol, of apparently polysaccharide material, which made photometric determination of DNA concentration impossible. This was removed by redissolving the pellet in water, and adding 0.8 - 1.0 volumes ethanol, and centrifuging to remove the polysaccharide. Ethanol was then added to the supernatant to a concentration of 70% to precipitate the DNA. While some of the DNA was lost by this process, that remaining was of high purity.

Polysaccharide precipitation could sometimes be avoided by precipitating DNA with propan-2-ol instead of ethanol..

(iv). Determination of DNA Purity and Concentration.

An aliquot of the DNA to be determined was first electrophoresed on a 1% agarose gel, and stained with ethidium bromide to check for the presence of RNA. If this was present, the specimen was treated with RNAse A, re-extracted and re-precipitated.

Concentration was determined spectrophotometrically on a Beckman DU50 spectrophotometer. DNA was scanned between 230 and 330nm., as the shape of the absorbence curve gives an indication of the purity of the sample. Absorbance was measured at 260 and 280nm.

DNA concentration was determined according to the equation:

$$A_{(260)}/20 = \text{mg/ml DNA.}$$

Purity was determined by the ratio $A_{(260)}/A_{(280)}$. If this ratio was not close to 1.8, the sample was contaminated, and DNA estimation could not be performed.

Usual contaminants were proteins or phenol. The former could be eliminated by phenol/chloroform and chloroform extractions, the latter by chloroform or ether extraction. For elimination of polysaccharide contamination, see genomic DNA preparation above.

(v). Transformation of *E.coli.*

Electro-competent bacteria were prepared by the method of Dower *et al.* (1988), which involved washing bacteria free of ionic material in ice cold de-ionised water, and suspending the washed culture in approx. 0.2% of the original culture volume of sterile 10% glycerol.

Electroporation was performed using 40 µl of competent cells in cuvettes of path length 2 mm.. 2.5 KV pulses were delivered by a Bio-Rad Gene Pulser with capacitance of 25 mF and 200 ohm bypass resistance.

Bacteria were then transferred to SOC medium, and incubated with shaking, at 37^{0} C for 1 hr. They were then plated onto LB + ampicillin plates to select transformants.

(vi). Electrophoresis.

a) Agarose gel electrophoresis.

Analytical gel electrophoresis was performed on horizontal mini gels, using 1% agarose in TBE buffer, and TBE as the tank buffer.

Preparative gels were made using TAE buffer, both for gel preparation, and as tank buffer.

Gels were stained with ethidium bromide 1 µg/ml for 15 - 20 min, and viewed on a UV transillluminator (302 nm for analytical gels and 366nm for preparative gels.).

Electrophoresis was performed at constant current of 80 - 100 milliamps.

b) Acrylamide gel electrophoresis.

All acrylamide gels were 4% w/v acrylamide, prepared by diluting 40% stock solution 1/10 in TBE buffer. The solution was degassed for 10 min, filtered, and polymerised by the addition of 0.05% v/v TEMED and 0.25% v/v ammonium persulphate, 1g /10 ml.

Sequencing gels had 50% w/v urea added as a denaturant.

Analytical gel electrophoresis was performed on horizontal gels, 1 mm thick, prepared on a Bio-Rad PAGE gel former. They were pre-electrophoresed at 100V for 10-15 min and electrophoresed at 60 V. They were stained with ethidium bromide as above.

Electrophoresis of radio-labelled PCR products for autoradiography was performed on vertical gels 1 mm thick between two glass plates. These were pre-electrophoresed at 300V for 15 min, and electrophoresed at 250 V.

The plates were separated, and the gel was soaked on the plates for 10 min in 12% acetic acid to fix the DNA. Gels were then transferred to 20% ethanol for a further 10 min and dried on the plate at 80°C. DNA detection was by autoradiography on X-ray film.

Sequencing gels were vertical denaturing gels, 0.25 mm. thick. They were pre-electrophoresed at 15 watts for 15 min and electrophoresed at 25 watts for approx. 1-2 hr. They were washed and dried on their plates as above.

(vii). Southern Blotting and Hybridation. (Sambrook *et al*, 1989)

Briefly DNA was transferred from agarose gels to nylon filters, by hydrolysing the DNA with HCl, denaturing with NaOH, neutralising, and transferring to Hybond Nylon filters by overnight capillary blotting. The filters were dried at 80°C for 1-2 hr to fix the DNA.

The filters were then treated with pre-hybridisation solution at 42°C for 2-6 hr, and with hybridisation solution with added denatured, radio-labelled probe, at 42°C overnight.

Hybridisations were performed in a Hybaid oven in the bottles provided.

The filter was then washed twice for 1hr with 2x SSC/0.1%SDS at room temperature, and once for 1hr in 1x SSC at 65°C. Washed filters were autoradiographed on Fuji X-ray film.

(viii). Preparation of Radiolabelled Hybridisation Probe.

Probes were prepared using the Bresatec oligolabelling kit with a ^{32}P labelled ATP, following the manufacturer's instructions.

Briefly the method involved annealing random oligonucleotides to the denatured probe DNA, and extending with Klenow polymerase. The labelled probe was separated from unincorporated nucleotides on a Sephadex G-100 column, using a Geiger counter to identify the fractions.

(ix) Separation of DNA from Agarose Gels.

This was done using the Gene Clean kit, following the manufacturer's instructions.

Briefly, the agarose was melted in a potassium iodide solution. The DNA was bound onto a silica matrix, washed with an ethanol solution , and eluted into TE buffer or water.

(x). DNA Sequencing.

a). Amplification Sequencing protocol of Cooper *et al.* (1993).

A reaction mixture of 2.5 µl Taq sequencing buffer, 20 ng γP^{32} labelled forward primer (hot primer), 100 ng unlabelled primer(cold primer), 1-2 units of Taq polymerase and 10 ng template DNA was made up to 21 µl. This was aliquotted into 4 reaction tubes, 5 µl per tube abnd mixed with 2 µl sequencing nucleotides containing respectively ddGTP, ddATP, ddTTP, or ddCTP. These were overlaid with mineral oil to prevent evaporation, and subjected to 25 thermal cycles as follows.

Denaturation: 95° C for 60 sec; annealing : 60° C for 60 sec; extension: 72° C for 40 sec. On completion, reaction was stopped with 4 µl stop buffer.

Electrophoresis of the heat denatured product was on 4% denaturing gel, which was dried on the plate and autoradiographed overnight.

b). PRISM Cycle Sequencing.

This was a single tube, fluorescent dye-labelling dideoxy sequencing kit. Sequencing was performed according to manufacturer' instructions.

Briefly, the supplied reation mix comprised ddATP, ddCTP, ddGTP,ddTTP each labelled with a different fluorescent dye; unlabelled dATP, dCTP, dTTP and dITP together with Taq DNA polymerase in a TRIS/$(NH_4)_2SO_4$/$MgCl_2$ buffer. To 9.5 µl of this was added 1 µg double stranded plasmid template, 3.2 pmol primer and water to a

volume of 20 µl. The mixture was overlaid with mineral oil and subjected to thermal cycling as follows:

Denaturation	95°C, 60 sec.
Annealing	50°C, 40 sec.
Extension	60°C, 4 min.

After thermal cycling, the extension products were purified by phenol/chloroform extraction and the DNA precipitated with ethanol.

The DNA was electrophoresed and the gel analysed by computer scan. Analysis and electrophresis were carried out by the University of Queensland, Department of Biochemistry and Molecular Biology.

(xi). DNA Ligations.

a). PCR products were ligated using the Pharmacia "Sure Clone" kit, and following manufacturer's instructions. Briefly, the overhanging 3' A was removed with Klenow polymerase, and the PCR fragment was kinased. The fragment was then cleaned in a spin column, and ligated into a pUC 18 vector, which had been cut with Sma I. and phophatased

b). Other ligations were performed in ligation buffer with 0.5 mM ATP, and 2 µl of T4 DNA Ligase per 10 µl of reaction mix, overnight at 15°C.

2.2. PCR MATERIALS. AND METHODS.

2.2.1. MATERIALS.

Note. Early preliminary work was done using the Du Pont Replinase kit. This used a *Thermus flavus* heat stable DNA polymerase. When this was withdrawn from the market, further work was carried out using *Thermus aquaticus* (*Taq*) polymerase for DNA amplification. All experiments reported in this thesis were carried out using Bresatec *Taq* polymerase.

(i). *Taq* (*Thermus aquaticus*) DNA polymerase was supplied by Bresatec at a concentration of 5.5u/µl, and diluted to working stock of 1u/µl, using the dilution buffer supplied by the company.

(ii). 10 x Reaction buffer was supplied by Bresatec, or made as required.

0.67 M Tris HCl pH8.8
0.166 M $(NH_4)_2SO_4$
4.5% Triton X-100
2mg/ml gelatin

(iii). Mg Cl_2 25mM was supplied by Bresatec, or made as required.

(iv). Nucleotides, dATP, dCTP, dGTP, dTTP were supplied by Boehringer.

(v). Light mineral oil was supplied by Sigma.

(vi). Oligonucleotide synthesis reagents were supplied by Pharmacia.

2.2.2. METHODS.

(i). Primer design.

Primers sequences were selected from known DNA sequences. The DNA sequences were checked, using the "CSIRO" program, for internal stem loops. As far as possible, sequences for PCR amplification were selected free of secondary structure, and sites of stem loops were definitely avoided as primer sites.

The JROLIGOS program (Rozas 1991) was used to find the free energy (ΔG) of primer-template dissociation, and to check for internal complementarity within each primer sequence and also for primer-primer complementarity. Primer pairs were selected to have approximately equal ΔGs, and to be non-complementary, both within their own length, and between each other.

(ii). Primer Synthesis

Primers were prepared on a Pharmacia Gene Assembler, de-protected by heating at 70°C for 1hr in concentrated ammonia solution and purified on a Pharmacia NAP column, according to manufacturer's instructions Concentrations were determined photometrically on a Beckman DU50 spectrophotometer according to the formula:

$$A_{(260)}/25 = \text{mg/ml Primer DNA}$$

(iii). Primer End Labeling .

5µl γ^{32}P ATP, 4 µl 20 µM primer, 1µl One Phor All buffer (Pharmacia), and 1 unit T4 polynucleotide kinase were incubated at 37°C for 30 min. The enzyme was heat denatured at 70°C for 5 min, and the labelled primer diluted 1/3 with unlabelled 20 mM primer before use.

(iv). Reaction Mixture.

PCR was carried out in 20 µl volumes in 0.5 ml microcentrifuge tubes, in a standard reaction mixture containing:

Tris HCl 67 mM pH 8.8,
16.6 mM $(NH_4)_2$ SO_4,
0.45% Triton X-100,
0.2mg/ml gelatin,
*$MgCl_2$ 2.5mM,
dATP, dCTP, dGTP, dTTP each 0.25 mM,
primers at 1-2mM,
1u *Taq* polymerase.

*Mg Cl2 concentration needed to be optimised for each primer pair, but unless otherwise stipulated, this was the concentration used.

All reagents except template DNA were pre-mixed and aliquoted into the reaction tubes, and covered with 2 drops (~ 20 µl) light mineral oil, to prevent evaporation during the reaction. Template DNA was added and centrifuged down through the oil just prior to thermal cycling.

(v). Thermal cycling.

A Bartelt Gene Machine was used for thermal cycling. Reaction tubes were cycled through three water baths set at different temperatures for denaturation, primer annealing, and polymerisation or extension. The water in each bath was overlaid with a layer of light oil to prevent the evaporation of the contents.

Denaturation was carried out at 95°C for 60-90 sec for plasmid DNA, for 3 min for genomic DNA, and for 5 min for whole cells. The extended times for genomic DNA and whole cells only applied to the first few cycles, after which the concentration of short PCR product outweighed that of the original template, and denaturation times were reduced back to those of plasmid DNA.

Annealing was carried out for 40-60 sec at a temperature dependent on length and base structure of primer according to the formula of Wu *et al.* (1991).

$$T = 22 + 1.43\ Ln$$

Where T is the annealing temperature, and

Ln is the number of bases in the primer plus the number of (Gs +Cs).

This formula gave the maximally stringent primer annealing temperature. For normal purposes, a temperature of about 5°C below the calculated temperature was found to give better product yield, while maintaining primer annealing specificity.

Polymerisation was carried out at 72°C. Time of polymerisation was approx. 1 min/Kb of target DNA.

Note. Times in each water bath included the ramp time required for the reaction to reach the set temperature.

2.2.3. CONTROL OF CONTAMINATION.

Because PCR is such a sensitive technique, a single copy of template DNA can yield an amplification product. Together with this sensitivity is the fact that the reaction produces many millions of copies of the original template, any one of which can itself act as template for a new PCR. In addition, the normal sources of DNA arising from routine laboratory activities such as plasmid preparation, genomic DNA extraction and so on may also provide contaminants.

There are three ways of avoiding contamination problems:

(i). By physical separation of reaction products and casual DNA from reagents and preparative areas e.g. Innes *et al.* (1990);

(ii). By production of a reaction product that is modified in some way so that it cannot act as a PCR template e.g. Cimino *et al.* (1991) or

(iii). By elimination of contaminating material from the reaction mixture by enzymic methods e.g. De Felippes (1991), Yu *et al.* (1991).

For an overview of all these methods, see Kwok and Higuchi (1990).

For this project, physical separation methods were used, which became more stringent as the full magnitude of the problem became apparent. Measures taken were as follows.

(i). All PCR reactions were prepared in a laminar flow cabinet, which was decontaminated with UV light between preparations. The laminar flow cabinet, all PCR preparative material and the thermal cyclers were placed in a room separate from the main laboratories. This room could be sterilised under UV, and was kept under positive pressure by means of a window fan drawing filtered air from outside.

(ii). All preparation of PCR template material was carried out in a different laminar flow cabinet in a separate laboratory. This cabinet was also sterilised by UV after each use.

(iii). Sterile gowns were worn over normal laboratory clothing, and surgical gloves used, for all PCR preparative work.

(iv). Each laminar flow cabinet was equipped with its own set of set of Gilson pipettes which were used only for the preparation of PCR reactions and reaction materials. All PCR reagents and template materials were manipulated either with a positive displacement pipette, or with normal Gilson pipettes fitted with filter tips. Pipette tips and microcentrifuge tubes were sterilised by autoclaving for 45 min at 105°C.

(v). All reagents capable of being autoclaved, namely, buffer, $MgCl_2$, distilled water and mineral oil, were sterilised at 115°C for 15 min. Nucleotides and primers which could not be autoclaved, were maintained specifically for PCR purposes, and prepared in sterile water in the preparative laminar flow cabinet.

(vi). Reagents were aliquotted into small volumes in closed tubes, so that if accidental contamination did occur, reagents could be discarded without undue cost..

(vii). Containers of preparative material were never opened outside their appropriate laminar flow cabinet.

(viii). Tubes containing amplification products were NEVER opened in the PCR preparation room.

(ix). Reagent blanks containing no DNA were run routinely with all PCRs , to check for contamination. If PCR products were detected i9n the absence of template DNA, which was not uncommon despite the above precautions, all reagents used in that reaction were disposed of, and new aliquots used for future reactions.