CHAPTER 5.

MISCELLANEOUS PCR TECHNIQUES.

PART 1. INTRODUCTION.

The main objectives for the present project were the development of PCR techniques for tracking and enumerating rumen bacteria. However, during the course of the project, other uses for PCR were developed, or became apparent. This chapter is concerned with five particular techniques that were found useful in the general area of rumen biotechnology, although they have application in the general fields of biotechnology and genetic ecology.

PART.2 EXPERIMENTAL WORK.

5.2.1. Investigation of Bacterial Transformations.

A quick screening test for successful transformants was developed, using PCR.

Materials and Methods.

After electroporation, transformants were plated onto selective media according to normal protocols. After overnight incubation, for *E. coli,* or three to five day incubation for rumen bacteria, colonies were harvested. The bacteria were either re-suspended in appropriate selective growth medium and grown to stationary stage, or the colony was suspended in 10-20 µl water. 100 µl of the stationary phase culture was centrifuged to pellet the bacteria, and the bacterial pellet was resuspended in 10-20 µl water.

The DNA template for PCR was 2 µl of the water suspended bacterial cells. The primers used were pUC Forward and Reverse primers (Promega), or suitable primers from

within the cloned gene, at an annealing temperature of 65°C for 40 sec. Twenty cycles of PCR were performed, with a 5 min. denaturation for the first cycle, and one min. thereafter. Extension times were dependent on the number of DNA base pairs between the primers, 40-60 sec. for less than 1 Kb, and correspondingly longer times for longer DNA templates. When the reaction was completed, 7-10 µl of reaction mix was electrophoresed on agarose gel and stained with ethidium bromide to visualise the PCR product.

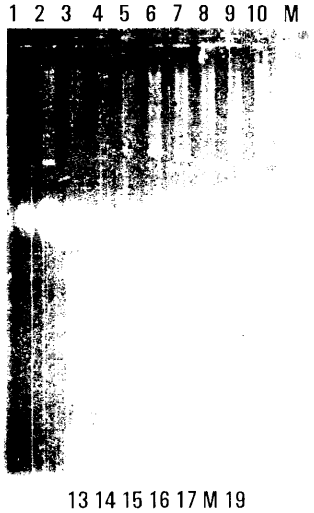
Results.

Figure 5.1. shows the results of using this technique to investigate the results of restriction fragments of genomic DNA from OB156 and AR29 "shotgun" cloned into pUC 18. [Chapter 3, Section 3.2.1a.(iii)]

Figure 5.1. PCR Amplification of Transformed Cells.

5.1(a).

AR29 Genomic DNA Transformants.



Lanes 1-10,13-17 and 19 show amplification products of AR29 genomic DNA transformants. Primers were pUC forward and reverse.

5.1.(b).

OB156 Genomic Transformants

Lanes 1-10,12-16 show amplification products of OB156 genomic DNA transformants Primers were pUC forward and reverse.

5.2.2. Construction of Chimaeric DNA Sequences.

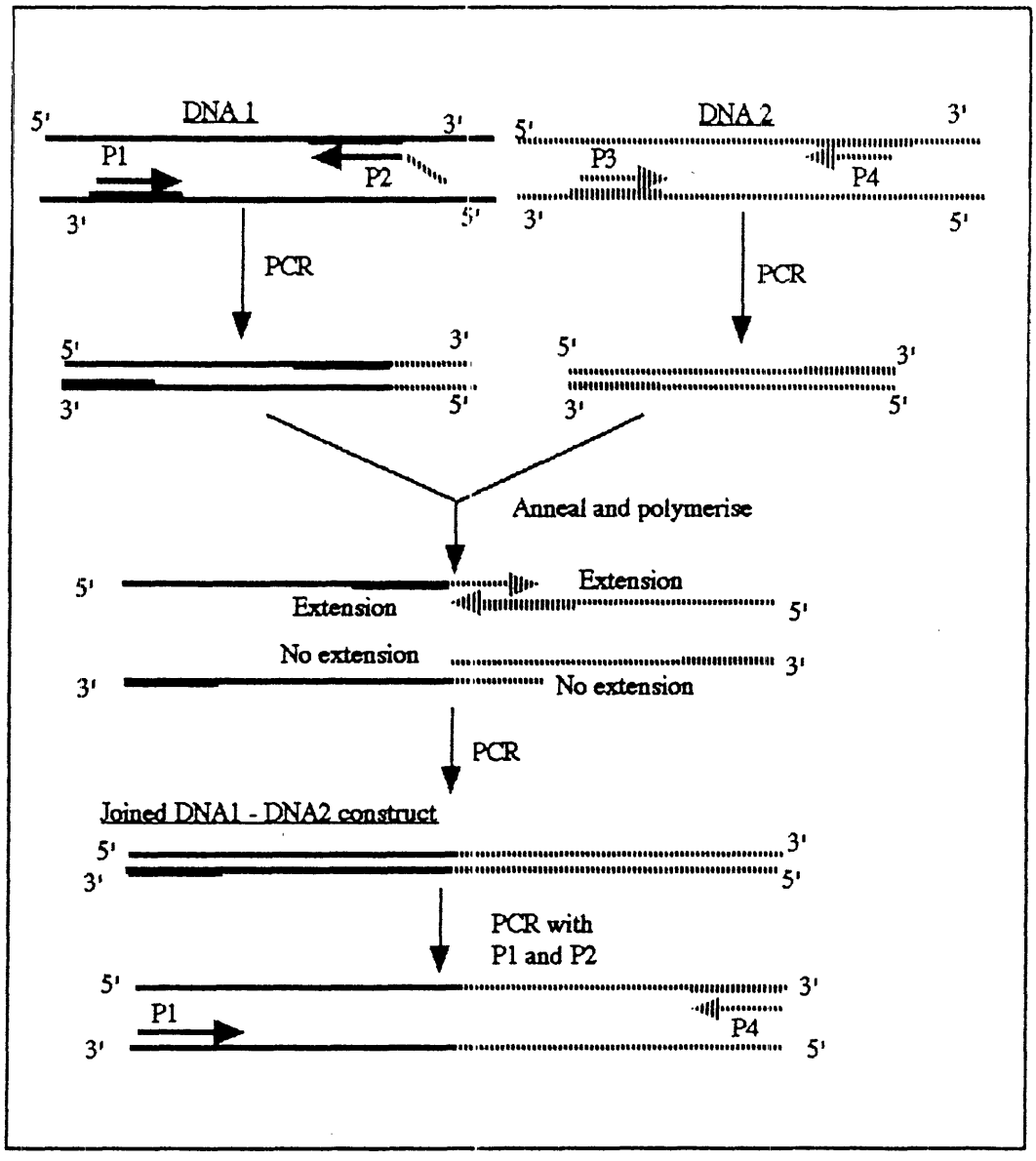
The polymerase chain reaction can be used to join two or more DNA sequences together. The technique overcomes the limits imposed by the use of restriction enzymes, because sequences can be cut at any point, independently of the presence of suitable restriction enzyme cutting sites. The technique was based on the work of Yolov and Zabarov, (1990). They demonstrated that by the use of overlapping primers, it was possible to unite two pieces of DNA from different sources. They termed the method "Recombinant PCR". The method is shown diagrammatically in Figure 6.1.

Briefly the two DNA sequences were amplified by PCR. However, the second primer for the DNA 1 sequence (P2 in the figure) had, in addition to its annealing sequence complementary to the 5' end of the upper strand of the DNA 1, a sequence complementary to the 3' prime end of the lower strand of the DNA 2. Thus, after amplification, the DNA 1 sequence had been provided with an overlap with DNA 2. DNA 2 was amplified separately with the P3 and P4 primers.

The two amplified sequences were then separated from their primers, and an aliquot of each placed in a PCR reaction tube. They were then heat denatured, and allowed to anneal. This resulted in two hybrid molecules, one with two 3' ends, which was unable to undergo extension, and the second with two 5' ends, each of which was able to prime polymerisation to the complete chimaeric DNA. This chimaeric molecule was further amplified using primers P1 and P4 to yield substantial quantities of the desired construct.

Figure 5.2.

Recombinant PCR.



Thickened sections of lines represent primer binding sites.
Primer P2 has overlap with DNA 2 sequence, which is incorporated into the amplification product. When the two amplification products are denatured and allowed to anneal, single strands from each anneal at the overlap. Only one of these hybrids can extend, as extension is 5' to 3'. Amplification of the chimera is increased by the use of P1 and P4 primers

(i). Signal Peptide - Dehalogenase Construct.

Recombinant PCR was used to add a signal peptide sequence to a dehalogenase gene.

The signal peptide - dehalogenase construct was wanted, because, while the *E. coli* plasmid pHS4 expressed the dehalogenase gene, the enzyme remained in the periplasmic space of *E. coli* and was not secreted into the culture medium. It was hoped that by the addition of the signal peptide to the 5' end, secretion of the dehalogenase into the medium would be achieved without loss of dehalogenase expression.

Materials and Methods.

Figure 5.3.

CelA Signal Peptide Sequence.

```
     GAT  TTTATGAGCA  CAAAATTCAT  CTGCTTGAGG  AATGtAATGG   360
    CTA  AAATACTCGT  GTTTTAGTCA  GACGAACTCC  TTACATTACC

CAATACTATT  GTCCATAAAC  ATTTTGTCCT  GTTCCAGTC  CAATTCCTC  419
GTTATGATAA  CAGGTATTTG  TAAAACAGGA  CAAGGTCAT  GTTAAGGAG
```

Primers.

1. P1 Primer PRSPA (5') TAGCTGACAAGCTTG**GATTTTATGAGCACAAAATT**
   Hind III **signal peptide start**

2. P2 Primer PRSPB (5') **TTGAATCCTGGAAAGTC**GAGGAATTGTACTGGAAC
   dehal start signal peptide tail

3. P3 Primer DehalF (5') GACTTTCCAGGATTCAAGAACAGC

4. P4 Primer Dehal T (5') AGATCAGGAAGCTTGCCTCTCTCTAGCGT
   Hind III **dehal tail**

Extra nucleotides were included at the 5' ends of the primers PRSPA and DehalT to provide *Hind III* sites. These sites were inset a few bases from the end to ensure efficient cutting. They were for use in cloning the construct, and a series of different primers could be used to provide different restriction enzyme sites. The two sites could be for different restriction enzymes if it was desired to directionally clone the construct.

The signal peptide was amplified using the primers PRSPA and PRSPB, and the dehalogenase gene using primers Dehal F and Dehal T. Template was pJW4 plasmid DNA for the signal peptide amplification, and pHS4 or pDS49 plasmid DNA for the dehalogenase gene.

PCR Conditions:

Denaturation:- 95 ^{o}C for 60 sec.

Annealing:- 55^{o}C for 40 sec.

Extension:- 72 ^{o}C, 40 sec for signal peptide, 90 sec for dehalogenase.

Cycles:- 25

$MgCl_2$ Concentration:- 2.5 mM for signal peptide. The dehalogenase gene amplification reaction proved particularly sensitive to Mg^{++} ion concentration in the reaction buffer. It proved necessary to amplify this gene at a $MgCl_2$ concentration of 1.25 mM, only half-normal concentration.

After amplification, the reaction product was electrophoresed on 1% agarose gel using TAE buffer, to separate the amplified product from its primers. The DNA was excised from the gel, and purified using Gene Clean. The resultant signal peptide and dehalogenase were used as template for recombinant PCR to produce the chimaeric signal peptide - dehalogenase construct, dilutions of the two sequences being adjusted to provide approximately the same number of template DNA molecules of each.

PCR Conditions:

Denaturation:- 95°C for 60 sec.

Annealing:- 55°C for 40 sec.

Extension:- 72°C for 90 sec.

Cycles:- 25

$MgCl_2$ concentration 1.75 mM.

Amplification products were electrophoresed on 1% agarose gels in TAE buffer. DNA was purified from the gel using Gene Clean or electro-elution, and the purified DNA cloned into pUC18, and sequenced to confirm the correctness of PCR amplified sequences.

(ii). Promoter - Dehalogenase Construct.

The dehalogenase gene had been successfully cloned into the *B. fibrisolvens* OB156 - *E. coli* shuttle vector, pBHerm, to give the plasmid pBHermF. The gene expressed in *E. coli* but failed to express in OB156. This was thought to be due to lack of a promoter sequence recognised by OB156. The erythromycin resistance gene was present on pBHerm, and successfully expressed, so its promoter sequence was used to form a chimaeric sequence with the dehalogenase gene.

Materials and Methods.

The promoter fragment was taken from the *Streptococcus faecalis* Eryth 1 gene. This gene was already present and known to express in the plasmid pBHerm. The primer Prom A contained nucleotides 1 - 17, and Prom B, nucleotides 95 - 112 of the Eryth I gene.

Primers.

1. P1 Prom A (5') TAGCCTGACAAGCTTAGAAGCAAACTTAA
   Hind III **Erm Promoter Start**

2. P2 Prom B (5') TTGAATCCTGGAAAGTC**CATGTAATCACTCCTTC**
   Dehalogenase start **Erm Promoter Tail**

3. P3 Primer DehalF (5') GACTTTCCAGGATTCAAGAACAGC

4. P4 Primer Dehal T (5') AGATCAGGAAGCTTGCCTCTCTCTAGCGT
   Hind III **dehal tail**

The promoter fragment was synthesised using Prom A and Prom B primers, with PCR conditions as for amplification of signal peptide fragment. The plasmid pBHerm was used as a source of template DNA.

The dehalogenase gene was synthesised using the primers DehalF and DehalT as above.

The promoter fragment and dehalogenase were co-amplified using Prom A and Dehal T primers under the same PCR conditions as the signal peptide and dehalogenase co-amplification. The construct was cloned into pBHerm, and sequenced to confirm the correctness of the PCR amplified sequences.

Results.

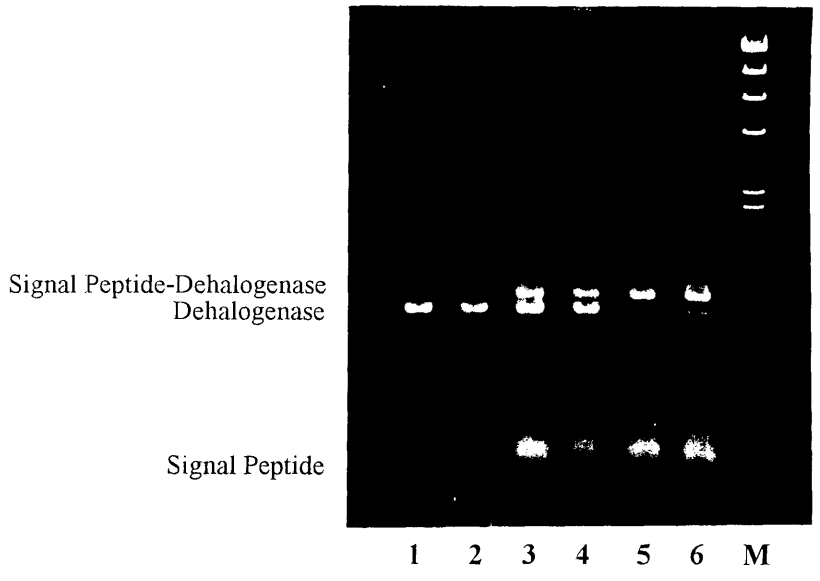
(i) Signal Peptide - Dehalogenase Construct.

Figure 5.4. shows the signal peptide and dehalogenase amplification products, and the results of the co-amplification of signal peptide and dehalogenase, using different numbers of PCR cycles. As can be seen, after 20 cycles the majority of the co-amplification product was the signal peptide - dehalogenase construct. However, usable quantities of product were available after 15 cycles, and it was preferred to use this

product for cloning, as the smaller number of replications reduced the chance of misincorporated bases.

Figure 5.4.

Recombinant PCR of dehalogenase Gene and Signal Peptide



Lanes 1 and 2 show products of PCR of AR20 signal peptide and dehalogenase gene after 10 cycles
Lanes 3 and 4 show products of PCR of AR20 signal peptide and dehalogenase gene after 15 cycles
Lanes 5 and 6 show products of PCR of AR20 signal peptide and dehalogenase gene after 20 cycles
Lane 7 shows λ phage/*Hind* III size marker

It is also apparent that some of the P2 primer remained after the fragment had been purified, as there was a considerable increase in the amount of signal peptide fragment in the reaction mix with increasing number of cycles. However, this did not interfere with the success of production of recombinant product.

The signal peptide dehalogenase construct was successfully cloned into a pUC 18 plasmid, and electroporated into *E. coli*. Sequencing confirmed that there were no misincorporated bases. However, the construct failed to express the dehalogenase gene in either a pUC18 plasmid in *E. coli*, or in the OB156 - *E. coli* shuttle plasmid pBHerm, in

the rumen bacterium *B. fibrisolvens* OB156. This was considered to be probably due to failure of the signal peptide to cleave from the enzyme.

(ii) Promoter Dehalogenase Construct.

The promoter - dehalogenase construct was successfully generated, and, after half end filling the *Hind III* site, cloned into the half end filled *Xba I* site on the pBHerm plasmid. Sequencing confirmed the correctness of the transcript. This plasmid pBHermF expressed the dehalogenase enzyme in the rumen bacterium *B. fibrisolvens* OB156.

5.2.3. 16S Ribosomal RNA Genes.

The work of Fox *et al* (1980), Woese *et al* (1985) and Pace *et al* (1986) has established the analysis of 16S ribosomal RNA as the basis for microbial phylogeny and taxonomy. This technique requires the isolation and sequencing of the 16S rRNA, or the genes from which it is transcribed. Less detailed information can be obtained from the studies of restriction fragment length polymorphisms (RFLPs) of the 16S rRNA genes. This was demonstrated by Moyer *et al* (1994) in a study of bacteria from a hydrothermal vent.

Various techniques, e.g. Hudman and Gregg (1989), Mannarelli *et al* (1988, 1991) have established that there is considerable genetic diversity within "species" of rumen bacteria. The comparison of 16S rRNA RFLP patterns seemed another possible method for examining this problem.

This work was done in consultation with Dr Brian Dalrymple of CSIRO Division of Tropical Animal Production.

Materials and Methods.

Primers.

These were taken from conserved areas at each end of the 16S rRNA genome, as given by Lane *et al* (1987)

1. 27F (5') GAGTTTGATCCTGGCTCAG
2. 1492R (5') ACGGCTACCTTGTTACGACTT

Template was either genomic DNA, or washed bacterial cells. Approximately 50 ng bacterial genomic DNA was used for each reaction. Bacterial cultures were grown to stationary phase, and centrifuged to pellet the cells. The cells were rinsed in water, re-centrifuged, and suspended in 5 x original volume of water. 5 µl of this suspension was used as template in each reaction.

The following strains of *B. fibrisolvens* were examined:

AR9; AR10; AR12; AR14; AR15; AR16; AR27; AR51; AR52; AR73; AR74, which were Armidale sheep *B. fibrisolvens* strains isolated by Dr Frank Hudman at the University of New England;

E 14, a reindeer *B. fibrisolvens* strain isolated by Dr Colin Orpin of CSIRO Division of Tropical Crops and Pastures;

OB156, a white tailed deer *B. fibrisolvens* strain isolated by Dr R. Forster of CFAR, Ottowa.

PCR Conditions.

Denaturation:- 95°C for 5 min for two cycles, then 60 sec for 28 cycles.

Annealing:- 60°C for 40 sec.

Extension:- 72°C for 90 sec

Cycles:- 30

Because maximum yields of product were required, large amounts of template DNA and many cycles of PCR were used.

After amplification 2-3 µl of product were electrophoresed on agarose gel to ensure that the reaction had succeeded, and that the product was the expected length, i.e. 1485 bp.

At first the amplified DNA was purified on Promega "Magic Prep" mini columns, but it was found that equally good results could be obtained by simply precipitating the amplified DNA with 2.5 volumes of ethanol. The precipitated DNA was digested with either *Hpa II* or *Rsa I* four base restriction enzymes according to manufacturer's instructions. After digest, the DNA was precipitated with 2.5 volumes of ethanol, and redissolved in 5 µl water, before electrophoresis on 2% agarose gel.

Results.

Restriction fragments of 16S rDNA are shown in Figure 5.5 for *Rsa I* digests, and in Figure 5.6 for *Hpa II* digests. Estimated lengths of the DNA fragments produced are tabulated in Table 5.2(a) and (b). It can be seen that these fragment lengths are only approximate, therefore the sum of fragment lengths varies to some extent from the true value of 1485 bp. Also, small restriction fragments less than about 50 bp were not visible.

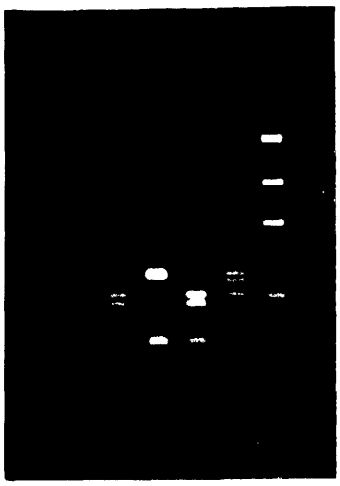
Figure 5.4

Rsa I Digests of *B. fibrisolvens* 16S rDNA

1 2 3 4 5 6 M 8 9 10 11

Lane 1, AR9; Lane 2 AR10, Lane 3 AR12
Lane 4, AR14; Lane 5, AR15; Lane 6, AR16
AR74;
Lane 7, Size marker, 2000, 1200, 800, 400, 200 100, bp.
Lane 8, AR27; Lane 9, AR51; Lane 10, AR52.
Lane 11, undigested 16S rDNA.

1 2 3 4 5 6 M

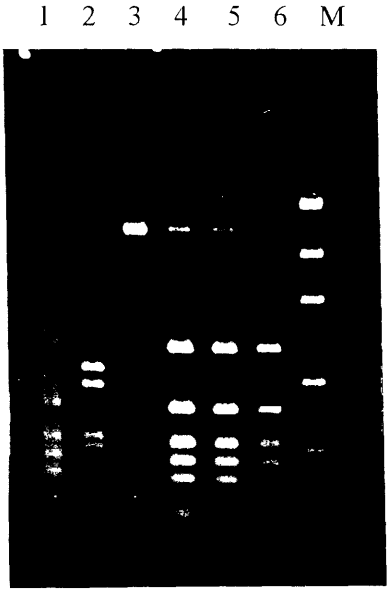


Lane 1, AR51, Lane 2, AR52;
Lane 3, AR73; Lane 4,

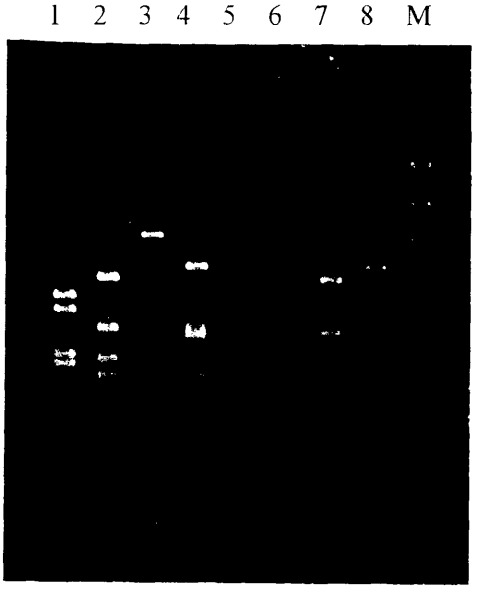
Lane 5, E14; Lane 6, OB156;
Lane 7, Size marker, 2000, 1200,
800, 400, 200, 100, bp

Figure 5.6

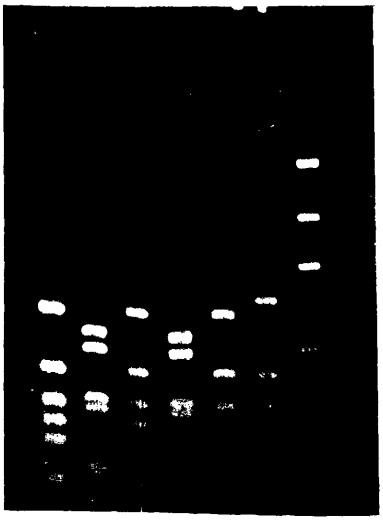
Hpa II Digests of *B. fibrisolvens* 16S rDNA



Lane 1, AR9; Lane 2, AR10;
Lane 3, uncut AR12 16S rDNA;
Lane 4, AR14; Lane 5, AR15;
Lane 6, AR16; Lane 7, size marker,
2000, 1200, 800 ,400, 200, 100, bp



Lane 1, AR12; Lane 2, AR27; Lane 3, AR51;
Lane 4, AR52; Lane 5, AR73; Lane 7, E14;
Lane 8 E14; Lane 9, OB156; Lane 10,.Size
marker, 2000, 1200, 800, 400, 200, 100, bp



Lane 1, AR9; Lane 2, AR10; Lane 3, AR73;
Lane 4, AR74; Lane 5, E14; Lane 6, OB156;
Lane 7, size marker, 2000, 1200, 800, 400, 200, 100, bp.

Table 5.1.(a). *Rsa* I digests

AR10 Type	E14 Type	OB156	AR51	E. coli
	80		80	
115				98
	120*		120	
	140		140	
165				
240	240		240	
	340			
	420	420	400	428
				457
480*		500	480	502
		540		
1480	**1460**	**1460**	**1470**	**1485**

Table 5.1.(b) *Hpa* II Digests

AR10 Type	E14 Type	OB156	AR51	AR52	E. coli
					5
80	80	80	80	85*	80
		90	90		
95			95		104
	110	110	110	110	109
					112
	140*	140	140	140	138
160	160	160	160	160	163
250	280	280		280	278
410					
470					496
	550	580		580	
			800		
1465	**1440**	**1440**	**1475**	**1440**	**1485**

Fragment lengths are in base pairs of DNA.
Figures in bold type show the sum of restriction fragment lengths.
* Shows apparent doublet bands.

Rsa I digest. Four groups of restriction patterns can be seen.

1 E14 Type:- AR9, AR14, AR 15, AR16, AR27, AR52, AR73 and E 14.

2 AR10 Type:- AR10, AR12 and AR74 (AR10 Type)

3 OB156.

4 AR51.

AR51 shows a similar pattern to the E14 group. It appears to lack one restriction site of that group, so that one of the 120bp fragments of the E14 group is combined with the 360bp fragment, giving a 480bp fragment in AR59.

The AR10 group has only the 120bp fragment in common with the E14 group.

OB156 has a restriction pattern totally different to any other group.

It is noticeable that AR52, which forms a separate group with *Hpa II* digestion, very similar to OB156, gives An E14 type restriction pattern with *Rsa I.*

There is no similarity between the *E. coli* restriction pattern and that of any of the *B. fibrisolvens* strains with digestion by *Rsa* I.

Hpa II digest. Five groups of restriction patterns can be seen:

1 E14 Type:- E 14, AR9, AR14, AR 15, AR16, AR27, AR73

2 AR10 Type:- AR10, AR12, AR74

3 OB156

4 AR51

5 AR52

OB156 and AR52 are similar except for differences in the shortest bands, and share the 110, 140, 160 and 280 bp bands with the E14 group.

AR51 is less similar to the E14 group. It appears to lack a restriction site present in the E14 group, giving a single band instead of the 580 and 280 bp bands of the E14 group, but possesses an extra restriction site which cleaves the longest band into fragments of approximately 800 and 95 bp.

AR10, AR12 and AR74 form a homogeneous group, with virtually no similarity to the other *B. fibrisolvens* groups.

E. coli restriction fragment lengths, taken from the restriction map of the published sequence, are included for comparison. It can be seen that the four fragment lengths, approximately 110, 140, 160, and 280 bp, common to three of the five *B. fibrisolvens* groups, are also present in *E. coli.*

5.2.4. Detection of Mismatched Primers.

The production of amplification product in the PCR reaction depends absolutely on the ability of the primer to anneal to its target DNA. The maximum annealing temperature is dependent on the energy of dissociation (ΔG) of the primer - template complex. This in turn is dependent on the primer length, and the number of matching nucleotides. Therefore the PCR system is able to determine mismatched primers by the presence or absence of specific amplification product at stringent annealing temperatures.

In order to obtain control sequences for *P. ruminicola* AR29 and *B. fibrisolvens* OB156, fragments of genomic DNA had been cloned into pUC18 (see chapter 3, p.3), and the inserts sequenced (See Appendix 1). The first primer sites selected (GOBF2 and GOBI for OB156, and GARF and GARI) were from close to each end of the readable sequence. When tested they proved to give no amplification product from their parent plasmids, pGOB5 and pGAR11 at the calculated maximum annealing temperatures (70°C for the pGOB primers, and 65°C for the pGAR primers). It was found necessary to reduce the annealing temperature to 60°C to obtain good yield of product from the GOB primer pair, and to 55°C for the GAR pair. However, at 60°C, the GOB primers proved non-specific for OB156. Interestingly, the GOB primer pair produced an amplification product from the *P. ruminicola* strains AR7, AR20, AR22 and AR23, slightly longer than that from OB156, (see fig 5.9.).

It seemed that at least one primer in each pair must be mismatched to its template by at least one base.

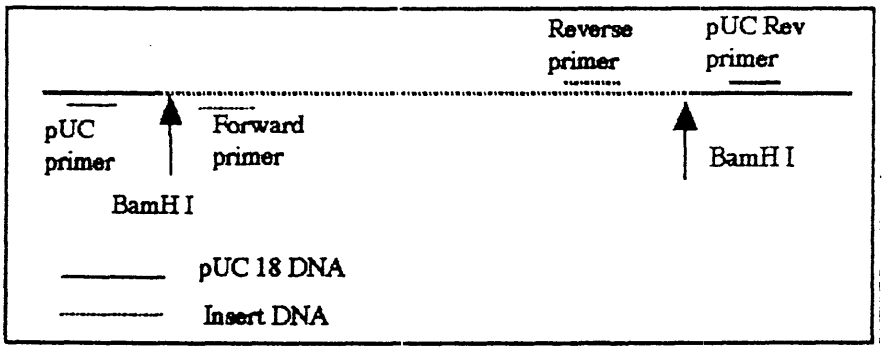
To determine which one of each primer pair was mismatched to its template, PCR reactions were performed using the forward primer and the pUC reverse primer, and the reverse primer with the pUC primer (see figure 5.7.).

Because the pGOB primer pair (GOBI and GOBF2) produced specific amplification product with the AR20 type *P. ruminicola* strains, the pGOB insert

sequence was checked with gene bank sequences to determine any homology with known sequences.

Figure 5.7.

Primer Sites for Determination of Mismatched primers.



The insert DNA is cloned into the *BamH* I site of the MCS of pUC18. The insert primers are "nested" within the sequence defined by the pUC primers. By using primer pairs of one pUC primer and one insert primer at high annealing stringency, it is possible to determine which insert primer is mismatched, as there will be little or no amplification product from the mismatched insert primer-pUC primer pair.

(i) Investigation of Mismatched Primers.

Materials and Methods.

PCR reactions were set up as shown in Table 5.3.

pUC primers were the Promega pUC/M13 24mer primers.

GOB primers were GOBF2 and GOBI

GAR primers were GARF and GARI

Primer sequences are shown in Appendix 1.

Table 5.2.

Templates and Primers for Determination of Mismatched Primers.

Template DNA	Primers
pGOB5 1ng	GOBF2 and pUC Reverse
pGOB5 1ng	GOBF2 and pUC Reverse
pGOB5 1ng	GOBI and pUC Forward
pGOB5 1ng	GOBI and pUC Forward
PGAR11 1ng	GARF and pUC Reverse
PGAR11 1ng	GARF and pUC Reverse
PGAR11 1ng	GARI and pUC Reverse
PGAR11 1ng	GARI and pUC Reverse

PCR Conditions.

Denature 95°C, 60 sec.

Anneal 65°C, 40 sec.

Polymerise 72°C, 40 sec.

Cycles 20.

5 µl of each completed reaction was electrophoresed on 1% agarose gel.

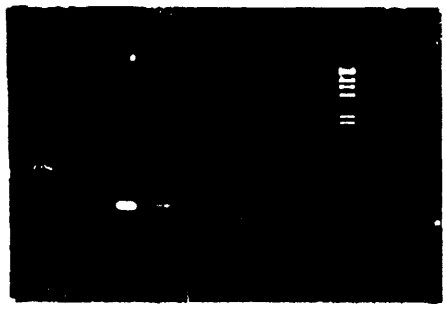
Results.

PCR results are shown in Figure 5.8. This shows that the GOBF2/pUC Rev, and the GARI/pUC primer pairs gave little or no amplification product, thus demonstrating that one primer out of each pair was mismatched, GOBF2 from the pGOB5 pair, and GARI from the pGAR11 pair.

Figure 5.8.

PCRs with pUC, pUC Reverse, and Suspect Primers.

1 2 3 4 5 6 7 M



Lanes 1 & 2 show products of pGOB with primers GOBF2/pUC Rev
Lanes 3 &4 show products of pGOB with primers GOBI/pUC
Lanes 5 & 6 show products of pGAR with primers GARF/pUC Rev
Lanes 7 & 8 show products of pGAR with primers GARI/pUC
Lane 9 shows λ phage DNA cut with *Hind* III as size marker..

It can be seen that with the GOBF2/pUC Rev primers, whose maximum calculated annealing temperature was 70°C, a small amount of amplification product appeared, but with the GARI/pUC primers, whose maximum calculated annealing temperature was 65°C, no product appeared.

(ii). Specificity of GOBF2 and GOBI Primer Pair.

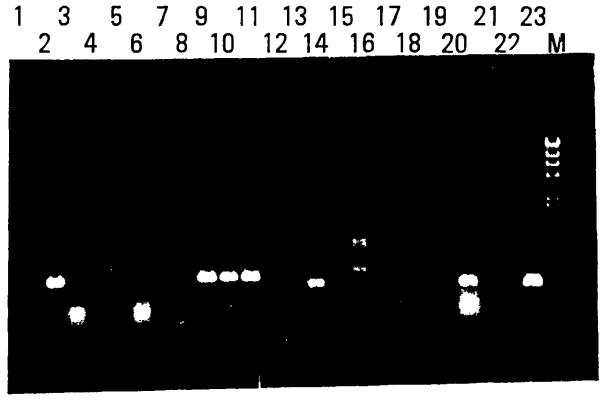
Materials and Methods.

The OB156 *B. fibrisolvens* GOBF2 and GOBI_primer pair was tested against a range of rumen bacterial DNA, as for Chapter 3, Section 3.2.1B.(i), with the annealing temperature set at 55°C.

Results

The amplification products from bacterial DNA using GOBF2 and GOBI_primers are shown in Figure 5.9. The amplification product from AR29*P.ruminicola* in lane 14 was demonstrated to be due to a contaminated DNA sample (results not shown). As can be seen from the AR29 amplification of lane 22, the GOBf2 and GOBI primers do not produce amplification product from AR29.

Figure 5.9.



Lane no	DNA Source	Lane no	DNA Source	Lane no	DNA Source	Lane no	DNA Source
1	AR3	7	#AR15	13	#AR27	19	AR53
2	*AR7	8	#AR16	14	(*AR29)	20	AR67
3	#AR9	9	*AR20	15	*AR30	21	OB156
4	#AR10	10	*AR22	16	*AR31	22	AR29
5	#AR12	11	*AR23	17	*AR33	23	pGAR11
6	#AR14	12	*AR24	18	AR36	24	pGOB5

All bacterial strains are identified in Chapter 3, p8.
* shows *B. fibrisolvens* strains. # shows *P.ruminicola* strains.
The amplification product in lane 14 is due to contaminated DNA.
Lane 25 shows λ phage/H*ind* III size marker.

The primer pair did produce a specific amplification product of the calculated length (351 bp) from *B. fibrisolvens* OB156, and a slightly longer amplification product from *P.ruminicola* strains AR7, AR20, AR21 and AR22. These four *P.ruminicola* strains are all genetically very close (see Chapter 3, p10)

The pGOB insert sequence (Appendix 1) proved to have 72.3% identity with part of the flagellin gene of *Roseburia caecicola*, and between 68.9% and 64.2% identity with flagellin genes from *Bacillus subtilis*. (See Appendix 2).

5.2.5. Production of Radio-Labeled Probes.

Radio-labeled DNA probes are used for a number of DNA hybridisation techniques. When the required probe sequence was present on a plasmid, the standard technique for radio-labeling was by annealing random oligonucleotides to the plasmid, and extending these fragments with DNA polymerase. Of the necessary deoxynucleotide triphosphates for this extension, at least one was labeled on the α phosphate. Thus the radioactive phosphate was incorporated into the extension products. Problems with this technique were that it labeled the whole plasmid, not just the sequence of interest, and that after reaction, the reaction mixture needed to be eluted through a column to isolate the labeled DNA from the oligomers and the unincorporated nucleotides.

By using PCR, probes could be produced in one of two ways. Firstly, the primer could be radio-labeled by kinasing with γP^{32} dATP. However, the amount of radioactivity incorporated by this method was not very great. Not all primer molecules were labeled in the kinasing reaction, and only one primer molecule is incorporated in each molecule of amplification product. Much more strongly radioactive amplification products can be achieved by using a ^{32}P dATP, or any other a ^{32}P labeled dNTP.

This method was used to make probes from the DS49top-DS49PE; DS63top-DS63PE; GOBF4-GOBI and GARF-GARI2 PCR target sequences to determine the copy number of these sequences on the genome.

Materials and Methods.

PCR reaction mixture was prepared as usual, except for the nucleotide mixture. Instead of using 0.25 µl each of dATP, dCTP, dGTP, dTTP per 20 µl reaction volume, a nucleotide mix was prepared comprising 2.5 µl each of dCTP, dGTP, dTTP; 1 µl dATP; and 6 µl αP^{32} ATP (Dupont). 1.5 µl of this mixture was used in each 20 µl reaction.

The reaction was performed with each primer pair and 1 ng plasmid DNA template. 30 cycles of PCR were performed, to give maximum yield of radiolabelled product.

Denaturation 60 sec, 95°C

Annealing 40 sec, 65°C

Polymerisation 40 sec, 72°C

After amplification was complete, 2 µl of product was electrophoresed on 1% agarose gel, and stained with ethidium bromide to ascertain the presence of the specific product.

The reaction volume was then made to 100 µl with water, to dilute out the PCR reaction buffer, and the DNA was precipitated with potassium acetate and 2.5 volumes of ethanol. The precipitated DNA was washed twice with water:ethanol 2.5:1 mixture, and the DNA was finally dissolved in 10 µl water.

Genomic DNA was digested with *Hind* III, which did not cut in any of the probe sequences, and electrophoresed on 1% agarose gel. The DNA was transferred by Southern blotting onto a Hybond nylon filter, and the probe was hybridised to the filter under standard conditions (Sambrook *et al,* 1989).

Results.

Figure 4.2.8. of chapter 4 shows the hybridisation results for the DS49top-DSPE sequence on the AR10 genome. All other PCR target sequences proved to be present at single copy number on their respective genomes (results not shown).

PART 3. DISCUSSION.

1. Investigation of Bacterial Transformations.

This method became routine in our laboratory, as it provided results more quickly and more easily than plasmid preparations followed by restriction enzyme digests. Initial screening could be done by Blue - White colony screening in some cases, when the inserts had been cloned into plasmids which had this option, or the alternative method of colony cracking was used in other cases. However, the successful transformants identified by this method could be screened for presence and length of insert by the PCR method. When, as was frequently the case with rumen DNA transformations, only a few transformants eventuated, all colonies were screened by PCR.

As well as determining the presence or absence of cloned sequences, the technique can be used to determine the direction of a cloned gene. This requires the use of two primers on the plasmid, such as the pUC forward and reverse primers, and at least one primer within the cloned gene. Two PCR reactions are set up, one with the forward primer, and the gene primer, one with the reverse primer and the gene primer. Only one of these reactions will produce amplification product, immediately determining the direction of the gene within the plasmid. This technique was used to determine the alignment of the promoter - signal peptide - dehalogenase construct in the plasmid pBHerm. (See p. 5.9.).

2. Construction of Chimaeric DNA Sequences.

This technique enabled sequences of known function, promoters and signal peptides to be grafted on to cloned genes to improve the performance of their expression products. The second chimaeric sequence produced had DNA from two separate bacterial sources. The dehalogenase gene was from *Moraxella species* and the promoter from *Streptococcus faecalis*. Moreover the construct was cloned into a third bacterial species,

B. fibrisolvens, in which the enzyme was successfully expressed. The technique provides for more accurate "cutting and splicing" of DNA, as primers can be designed to target and amplify any given piece of sequence, and the experimenter is no longer dependent on being able to find suitable restriction sites, which may not be present at the most desired positions.

Cloning problems, arising from the fact that PCR with *Taq* polymerase may produce a product with a 3' A overhang, may be overcome by removing the overhang before ligating the PCR fragment into the cloning vector. The Pharmacia "Sure Clone" kit was used in these experiments (see Chapter 2, p12).

3. 16S Ribosomal RNA Genes.

The production of amplified 16S sequences from a variety of bacteria by PCR proved to be an easy process, and restriction fragment polymorphisms could be readily identified. The RFLP patterns largely confirmed the DNA hybridisation results of Hudman and Gregg (1989) on the relationships between various strains of *B. fibrisolvens*, particularly the lack of relatedness between the AR10 group, and the E14 group. However, there were some strains that did not fit either group, and there was one strain, AR52 which appeared close to OB156 with *Hpa* II digestion, but grouped with E14 with *Rsa* I digestion. Also OB156, which shared several RFLPs with the E14 group under *Hpa* II digestion, appeared totally different from the E14 group under *Rsa* I digestion. It is therefore apparent that if this technique is to be used to group strains within bacterial "species", more than one restriction enzyme should be used, preferably several. Moreover, this technique can only be approximate. To determine true and accurate relationships between strains, it is necessary to fully sequence their 16S ribosomal genes, and analyse their sequences in comparison with known species.

The 16S rDNA RFLP technique does not provide such detailed phylogenic information as either genomic restriction patterns or DNA hybridisation. However, because it is quick and simple, the technique may have value as an initial screening test.

4. Detection of Mismatched Primers.

Mismatched primers were readily identified by PCR. This technique could be extended to other problems such as determination of difficult to read stretches of sequence. One technique for resolving these is to synthesise oligonucleotides complementary to each of the readings of the doubtful sequence, anneal the to the sequence, and determine the melting point temperature between oligonucleotide and sequence. With PCR, the same oligonucleotides could be synthesised, but they could be paired with a primer from an unambiguous section of sequence. The annealing temperature at which amplification product was formed would readily identify the most complementary oligomer, and hence the correct sequence.

5. Production of Radio-Labeled Probes.

Specific DNA probes with high level of intrinsic radioactivity could be easily produced by PCR. The method produced a more specific probe than the technique of extension from random oligonucleotides on plasmid DNA, as only the sequence of interest was labeled, rather than the entire plasmid.

In summary, a number of PCR techniques were developed for specific experimental purposes that proved to be valuable for a variety of biotechnological uses.

CHAPTER 6.

DISCUSSION.

Summary

This chapter considers the techniques, which have been developed, and the results which have been obtained, during the course of this project, and relates them to the work of other researchers in the fields of rumen ecology and biotechnology, as well work in other areas of environmental bacterial ecology. Future research directions suggested by the results of the present project are proposed.

6.1. Sampling, Template Preparation and Target Sequences.

The problems faced in developing PCR techniques for tracking rumen bacteria *in vivo* can be divided into those peculiar to the rumen bacterial ecosystem, and those which are inherent in the polymerase chain reaction. The PCR related problems include the extreme sensitivity of the reaction, which produces extreme sensitivity to contamination, and the limited amount of sample that can be processed in any one reaction. The contamination problems have been discussed in Chapter 2, section 3.2, and were reviewed by Kwok and Higuchi (1989). Briefly, contamination control was achieved by isolation and separation of sample preparation and reaction preparation, and by keeping sample manipulation to a minimum. Limitations imposed by sample size are due to the fact that PCR is, in essence, a micro technique, with a reaction volume limited to about 50 µl for practical purposes. Strategies for detecting bacteria at concentrations less than one cell per PCR reaction volume were briefly considered in Chapter 3, p 3-54, and will be further discussed later in this chapter.

Problems arising from the rumen bacterial ecosystem are related to the large numbers of rumen bacteria, their diversity, and the lack of uniformity of the rumen environment. These all have bearing on sampling strategies, template material and sequences selected for PCR probes.

(i). Sampling Strategies.

Bacterial distribution is not uniform throughout the rumen. There are three major bacterial habitats within the rumen. The first is the ingested plant material, which is heavily colonised by fibrolytic bacteria, many of which adhere very strongly to their substrates. The best methods of dissociation are only able to detach about 80% of particle associated bacteria (Whitehouse *et al,* 1994). The second is the liquid rumen fluid phase, which favours bacteria relying on soluble substrates. The third is the rumen epithelium.

In addition to bacteria, anaerobic fungi and protozoa are present in the rumen in significant numbers, and, being eucaryotic, have much larger genomes than bacteria. The cellulolytic fungi attach to plant fragments, but the protozoa are free swimming.

The above factors needed to be taken into consideration when devising strategies for sampling rumen contents. In addition, since PCR was used as the tracking method, the extreme sensitivity of PCR, and consequently the likelihood of false positive results from any trace of contaminating target DNA, meant that it was desirable to keep sample manipulation to a minimum.

It was not possible to devise a simple sampling strategy that would give a representative sample of the bacteria colonising the rumen epithelium, so these bacteria were ignored. The bacteria under investigation for this study were xylanolytic, and could therefore be expected to be found predominantly attached to plant material. However, since these bacteria need to be able to move between food fragments, part of the population could be expected to be found in the liquid phase. This was confirmed for *B. fibrisolvens* AR10 [Chapter 3, section 2.2. (ii).], using rumen samples containing small (<1 mm diameter) plant fragments.

Therefore, in order to obtain reasonably representative samples both plant material and rumen fluid were required. Without going to the extent of totally emptying the rumen, homogenising the contents, and sampling the homogenate, samples taken would not reliably give a true estimate of total rumen bacterial numbers of both free and attached bacteria. The best that could be done was to take a sample that was reasonable reproducible and representational of both bacterial populations. For this project, only small plant fragments in suspension in the rumen fluid were sampled, which gave a suspension that could be used (after washing) directly for PCR template, and still included a proportion of attached bacteria. This was in contrast to Stahl *et al* (1988) and McSweeney *et al* (1993) who included large plant material, and homogenised it for

template preparation. They considered (McSweeney, pers. comm.) that this would give a more representative sample of the bacterial population, and were apparently not concerned about extra preparation steps, possible contamination and reproducibility of sample. For this project, the inclusion of large plant fragments in samples to be tested was considered to over-complicate the sampling method. This was because there seemed no way to determine how much large plant material would be representative of total rumen contents, or how to reproduce this amount from sample to sample, and because the extra homogenisation step increased the chances of sample contamination.

Samples for tracking purposes, where the animals had been rumen fistulated, were taken from the predominantly liquid portion of the rumen contents, below the fibrous "raft" of large plant fragments. The fistulated sheep used for the tracking experiment were fed hourly throughout the day and night, and, as far as could be determined by observation, ate each food ration as it was delivered. Water was provided ad lib. This provided a reasonably constant rumen environment for sampling. Samples were taken at about the same time each day. While these samples were not necessarily representative of the total rumen population, they were considered to comprise a reasonably reproducible subset of the rumen population.

Field samples were taken via mouth or nose tube inserted down the oesophagus into the rumen. The material sampled, which included plant material up to about 5 cm long, was then sub-sampled, after mixing, with a Pasteur pipette to exclude plant material over ~1 mm diameter. This material then represented a sample of small plant material suspended in rumen fluid, similar to the material from fistulated animals. However, since food and water intakes were neither monitored nor controlled, these field samples represented more random samples of the rumen population.

(ii). Template material.

Rumen genetic material to be tested could take the form of extracted nucleic acids, e.g. Stahl *et al* (1988), or Attwood *et al* (1988), who used nucleic acid hybridisation techniques, or whole cells, e.g. Amman *et al* (1992), who used fluorescent rRNA probes. In PCR techniques for studying non-rumen bacteria, Steffan and Atlas (1988), Chaudry *et al* (1989), Bej *et al* (1989), used extracted DNA, and only Joshi *et al* (1991) had demonstrated the use of whole cells as PCR template material.

A disadvantage of using extracted nucleic acids is the impossibility of separating bacterial nucleic acids from other contaminating genetic material from plants, fungi and protozoa. Stahl *et al* (1988) admitted this when they were unable to determine a meaningful figure for bacterial numbers from probes hybridised to total rumen ribosomal RNA, because they were unable to estimate the proportion of RNA which was of non-bacterial origin. The present study used extracted DNA only for the detection of the presence of bacteria in North Queensland field samples (Chapter 3, section 2.3). Also the DNA was extracted after lysozyme disruption of the cells, which should favour extraction of bacterial DNA over plant and fungal DNA. This was in contrast to total rumen DNA extraction, Attwood et al (1988), or total rumen RNA extraction from mechanically disrupted rumen cells, Stahl *et al* (1988), and McSweeney *et al* (1993).

Another disadvantage of using extracted DNA is that DNA extraction techniques are time consuming, and increase the chance of contaminating the samples. The use of whole cells as PCR template, adapted from the method of Joshi *et al* (1991), eliminated the process of extracting DNA from rumen samples, and removed any concerns that the extracted DNA might be biased in favour of more readily lysed bacterial strains. It also considerably lessened the chances of contamination by reducing the number of steps in preparation of the PCR template. The use of strain-specific target sequences, whose copy number on the genome was known, made quantitation of cell numbers in rumen samples relatively simple.

Wang *et al* (1992 and 1996)also developed methods using whole cells as PCR template material for the determination of *Listeria monocytogenes* in food (1992), and for the detection and quantitation of a variety of bacterial species in faecal samples (1996). In both cases the template cells were prepared from food or faeces by simple washing and centrifugation steps. They were prepared for PCR by boiling in 1% Triton X-100 for 5 minutes, and then cooling before being added to the PCR reaction mix. The process used in this project differed in that Triton X-100 was incorporated in the PCR reaction mix, and the whole reaction mix, including template cells, was heated at 95°C for 5 minutes for each of the first few cycles, to permeabilise the cell membranes. This provided a slightly simpler process than that developed by Wang *et al* (1992).

(iii). PCR Target Sequences.

PCR primers can be designed to target any sequence. Steffan and Atlas (1988) used an unidentified 1.3 Kb repeat sequence for tracking *Pseudomonas cepacia*; Chaudry *et al* (1989) transformed their *E. coli* strain with an unidentified plant DNA target sequence; Bej *et al* (1989) and Joshi *et al* (1991) used known gene sequences for their PCR probes.

Cloned and sequenced endoglucanase genes were used as target sequences for *Prevotella ruminicola* AR20, and for *Ruminococcus albus* AR67. However, for the other target strains, *Butyrivibrio fibrisolvens* strains AR10 and OB156, and *P. ruminicola* AR29, little sequence information was available.

There were difficulties with cloning most rumen bacterial DNA into *E. coli*, discussed by Gregg (1992). The problem was demonstrated by the small number of colonies produced from "shotgun" cloning OB156 and AR10 genomic digests [Chapter 3, Section 3.2.1 (a)], and the lack of success in cloning deletant DNA fragments for PCR control sequences [Chapter 4, pp 4 and 20). Because of this, no attempt was made to identify gene sequences for AR10, AR29 and OB156 for use as PCR target sequences. Instead it was decided to use randomly cloned genomic material and rely on the specificity conferred by the use of 21mer - 24mer primers. Primers targeting these random genomic sequences proved strain specific for two target sequences for AR10 and single target sequences for each of AR29 and OB156 (Chapter 3, pp9-11 and 37)

Other researchers in rumen microbiology have not used PCR for tracking rumen bacteria, so there are no comparative data on choice of PCR target sequences for rumen bacteria. However, PCR has been widely used in the detection and enumeration of bacteria in other environmental material. While there is too much published material to be considered in detail, relevant examples can be cited.

The most commonly used target sequences are from 16S rRNA (e.g. Wang et *al*, 1992 and 1996; Klijn *et al*, 1995). An advantage of using 16S rRNA sequences was that they also provided phylogenic information. As 16S rRNA sequence is an important determinant of bacterial phylogeny (Weisburg *et al*, 1991; Ludwig and Schleifer, 1994) it makes sense to use target sequences that define the species, subspecies or strain that is to be targeted. Researchers who targeted specific gene sequences did so because they were more interested in the distribution of the gene than the distribution of the bacterial strains,

as was the case with Szabo *et al* (1994), who tracked *Clostridium botulinum* strains using PCR primers targeted to the botulinum neurotoxin genes.

The choice of a strain specific target is related to the whole area of bacterial evolution and classification. Because different parts of the genome mutate at different rates (e.g. "conserved", "variable" and "hypervariable " sequences), and because genetic material is well known to be transferred between bacterial species, it is very hard to predict unique target sequences that will truly delimit one strain from another, unless, as with 16S rRNA sequences, the strain can be defined by the sequences used. While the use of target sequences from known genes and random genomic DNA fragments proved satisfactory for the purposes of this project, and had the merit of being easily prepared, it is considered that future tracking work should seriously consider using strain specific 16S rRNA target sequences. This would also have the advantage of correlating the phylogeny of different bacterial strains isolated and characterised by different research groups.

The limit of detection of one bacterial cell per microlitre of rumen sample imposed by the techniques used in this experiment is at least partly due to the very large number , $\sim 10^{10}$/ml (Stewart and Bryant, 1988) of bacteria in the rumen. Excess DNA reduces the sensitivity of PCR (see Chapter 4, p 33) and sensitivity of detection of cells in rumen samples is only one quarter that of those in water (Chapter 3, pp 15-16).

There are various ways by which this detection limit could be improved. An order of magnitude in sensitivity could be gained by running multiple amplifications of single samples. Statistical analysis of the number of reactions which produced amplification product would give an estimate of cell numbers in the sample. Selective sampling of plant material without liquid rumen contents would enrich the sample with fibrolytic bacteria. Similarly, sampling of the liquid phase only would bias towards the non-fibrolytic species. The third possibility is the use of enrichment media. At present, media specific for all strains of *Butyrivibrio* or of *Prevotella* have not been defined. Rumen fluid medium with xylan as the sole carbon source would select for both species. However, the development of defined media for the various target strains would not only improve enrichment strategies, but assist in determining phenotypic characteristics which would help define the ecological niches occupied by these strains within the rumen ecosystem. The desirability of this is stressed by Lin *et al* (1994) with respect to *Fibrobacter*. Defined nutrient requirements for particular strains would lead to the possibility of altering rumen

conditions to favour particular strains, or, conversely, introducing particular strains into the rumen to improve ruminant nutrition with different feed intakes.

6.2 Quantitation Techniques.

PCR has the advantage over DNA hybridisation techniques in that it is readily quantifiable. Experimental results showed (Chapter 4, Section 4.2.4) that if templates contained small numbers of bacterial cells, about 10 cells or less per PCR reaction, it was possible to estimate the cell numbers in the PCR reaction to within $\pm$ 1 or 2 cells, either by competitive or non-competitive PCR, with competitive PCR giving slightly more accurate results. An accuracy of $\pm$ 1 or 2 x 10 is predictable from amplification of samples containing up to 100 cell/µl.

In comparison, Stahl *et al* (1988), McSweeney *et al* (1994) and Lin *et al* (1994) all used species specific or strain specific 16S rRNA probes, hybridised to total rumen RNA, which could not give an accurate estimate of bacterial numbers.

McSweeney *et al* (1993), tracking *Synergistes jonesii,* and Lin *et al* (1994) tracking *Fibrobacter* species had some success in circumventing this problem by using both a general eubacterial 16S rRNA probe, and a probe specific for the bacteria they were tracking, and estimating the abundance of probe specific 16S rRNA as a proportion of eubacterial 16S rRNA. However, for actual quantitation of bacterial numbers, it would be necessary to know both the ribosome numbers in their target bacteria, and the average ribosomal count of all other eucaryotic material, a calculation complicated by the fact that ribosomal numbers vary with the growth stage of the organism (Amman *et al,* 1992).

Quantitation by selective plating of antibiotic resistant mutants has been demonstrated by Flint *et al* (1988) to track *Selenomonas ruminantum* and *Bacteroides multiacidus*, and by Miyagi *et al* (1994) to track *Ruminococcus albus.* However, although these methods give useful information for the particular mutant strains, they cannot be used in general to track normal bacteria without usable resistance markers. Sharp *et al* (1994) monitored survival of *Lactobacillus* strains within the rumen, and estimated total *Lactobacillus* by a combination of selective plating, and aerobic culture. By culturing aerobically, they were able to select against the majority of rumen bacteria, which are strictly anaerobic. However, this is hardly a useful approach for the study of most rumen bacteria.

Competitive PCR does not seem to have been used for the quantitation of environmental bacteria. The preferred PCR quantitation technique used, exemplified by Wang *et al* (1996), was serial dilution of samples prior to amplification, and determination of the lowest dilution in which amplification product can be detected. Tenfold dilutions were commonly used, which gave cell counts to within an order of magnitude. However, if greater accuracy were required, smaller dilution factors could be used. This procedure eliminates the need for densitometry scanning, but requires the amplification of much larger numbers of samples than does competitive PCR.

6.3 Results of Tracking and Quantitation Experiments.

Several strains of rumen bacteria were introduced into the rumens of live animals, and their persistence in these animals tracked over long periods. Quantitation of these strains in defined rumen samples was achieved, and changes in population of each strain within the rumen in response to different diets were demonstrated. These results are considered in relation to overall rumen bacterial populations.

(i). Rumen bacterial genetics and populations.

When considering numbers of particular bacterial strains within the rumen, it was necessary to take into consideration recent advances in the understanding of rumen bacterial genetics and phylogeny.

Phenotypically related bacteria may be genetically unrelated, and the number of genetic strains comprised by a single phenotypic species may be very large. Work on the rumen bacterial species *Prevotella (Bacteroides) ruminicola* Avgustin *et al* (1994) and Paster *et al* (1994); *Butyrivibrio fibrisolvens* Foster *et al* (1996) and Willems *et al* (1996); *Ruminococcus albus* (Rainey and Janssen, 1995); and *Fibrobacter succinogenes* (Lin *et al*, 1994) has extended and confirmed the work of earlier researchers (Chapter 1, section 3), demonstrating that these "species" actually consist of multiple clusters of related strains with, in many cases, little genetic relationship between the clusters.

Standard evolutionary theory strongly suggests that each of the many diverse bacterial strains within each phenotypic species occupies its own niche within the rumen ecosystem, and has its own unique requirements, which ensure its competitive advantage under defined environmental conditions. Because the ecosystem as a whole has to be

adaptable to a range of nutritional intakes, and the bacterial population is subject to predation by protozoa (Williams and Coleman, 1988), and attack by lytic bacteriophages (Klieve *et al*, 1991), at any one time only a proportion of strains is likely to meet favourable conditions. The rest are likely to remain "dormant", i.e. present at very low levels, but able to reproduce very rapidly to significant levels whenever conditions favour them. This hypothesis was supported by studies on levels of mimosine degrading bacteria (Hammond *et al*, 1989), showing that mimosine degrading bacteria could not be detected in the rumens of cattle until *Leucaena* was introduced into the diet, when the population rose to significant levels over a period of about 10 days. Results obtained from the introduction of bacteria into sheep rumens (Chapter 3, Section 2.5), particularly those from *P. ruminicola* AR29 in sheep no. G8 (Chapter 3, Graph 3.2.6) clearly show the ability of bacterial strains to remain at levels of less that ~10^3/ml of rumen sample for several weeks, before rising to significantly higher levels in response to dietary change.

Because there are no data on the number of strains of any one phenotype that may be present in the rumen of a single animal, nor how many strains may be expected to be "dominant" at any one time, the level at which successful strain may be maintained in the rumen under favourable conditions can only be conjectured.

(ii). Population Levels of Introduced Strains of Rumen Bacteria.

Of the introduced strains studied in this project, the most successful was *B. fibrisolvens* OB156, which was able to maintain a population of ~4 x 10^5 - 4 x 10^6/ml of rumen sample in one sheep (Chapter 3, Graph 3.2.8) This, represents 0.04 - 0.4% of the rumen bacterial population, taking the rumen bacterial population as ~ 10^{10}/ml (Stewart and Bryant, 1988). However, *Butyrivibrio* phenotypes may make up 26% or 30% of the rumen population (Forster *et al*, 1996). Even assuming that *Butyrivibrio* phenotypes represent 20% of rumen bacteria, then, at best, OB156 comprised only ~2% of the *Butyrivibrio* population. Therefore, either OB156 was not a markedly successful coloniser, or, if this is a typical level for a successfully dominant strain, it would have to be assumed that there were at least 75 -100 strains of *Butyrivibrio* in the rumen. This figure allows for between one third and half the strains present to be "dormant". Although these figures are speculative, they do indicate the possible presence in a single rumen population of many more strains of *Butyrivibrio* phenotypes than had previously been assumed.

The level of OB156 was the highest population level achieved by any of the strains introduced into sheep rumens *in vivo*. *B. fibrisolvens* AR10, and *P. ruminicola* AR20 remained at levels of ~ 10^4/ml and *P. ruminicola* AR29 at levels of ~ 10^5/ml.

It should be emphasised that these low level "dormant" strains are as important to rumen ecology as the temporarily dominant strains, as they provide the resources whereby the ruminant can readily adapt to changing conditions of nutrient intake.

6.4. Introduction and Maintenance of Bacterial Strains in the Rumen.

All bacterial strains introduced into the rumen during the course of this project were maintained in the recipients over long periods, albeit at low population levels that periodically fell below the limit of detection (~10^3/ml). The introduction of *B. fibrisolvens* AR10 into cattle under field conditions showed that not only was it possible to introduce this strain, isolated from sheep rumen, into cattle in an area where the organism had been previously undetected (Chapter 3, Section 3.2.3), but that the organism was able to spread through the herd (Chapter 3, Section 3.2.4). These results from Headingley station also demonstrated that AR10 fell below the limits of detection in over 90% of cattle tested at day 268 under conditions that were apparently adverse to it, but was later detectable in over 30% of cattle tested at day 404 when conditions had improved.

The experiment with inoculation of four strains of rumen bacteria, including one genetically modified strain proved equally successful. *B. fibrisolvens* AR10 was already present in one, and possibly both sheep, but the other three organisms also maintained populations in both sheep tested over a period of 149 days. All strains in both sheep, which were kept isolated from each other to prevent transfer of bacteria from one to another, fell below detectable limits at one time or another. The genetically modified strain, *B. fibrisolvens* OB156, which originated from a Canadian white tailed deer, maintained itself more consistently, and at higher levels than the three strains which had been isolated from Armidale sheep, *B. fibrisolvens* AR10, and *P. ruminicola* strains AR20 and AR29. This was in spite of the fact that its genetic modification, a plasmid bearing the fluoroacetate dehalogenase gene, seemed extremely unlikely to confer any selective advantage under normal rumen conditions.

The population levels of introduced strains discussed above have bearing on problems that have been reported with returning laboratory cultured strains of bacteria to the rumen. Attwood *et al* (1988) reported failure to introduce *P. ruminicola* B_14 to the rumen, but their lower limit of detection was 10^7 cells/ml. The results obtained in this project suggested that this is a level which could be difficult to attain, and that the failure of B_14 to establish could have been more apparent than real. Flint *et al* (1989) had successfully introduced and maintained *S. ruminantium* strain F100 at a level of ~10^6 for thirty days, but had reported failure with another strain, *S. ruminantium* SS2/R5, and with an isolate of *Bacteroides multiacidus*. Sharp *et al* (1994) introduced both an unmodified and a recombinant strain of *Lactobacillus plantarum* into the rumen of sheep, and showed that neither could be detected after 24 hours. The limit of detection appeared to be ~ 10^3/ml. They demonstrated that the rapid loss could be due to protozoal predation. The most successful reported introduction of bacteria into ruminants was the transfer of the *Leucaena* detoxifying bacterium, *S. jonesii,* from Hawaiian goats into Australian ruminants (Jones and McGarrity, 1986).

Another factor that needs consideration is the very rapid decline in population levels of introduced bacteria immediately after inoculation that was shown by all bacterial strains tested except for AR10. Since this strain was already present in at least one of the experimental sheep, this discrepancy is readily explained. Other strains took several days to establish themselves after inoculation, and then numbers fluctuated considerably. This decline in numbers after inoculation was corroborated by McSweeney *et al* (1993) with Synergistes *jonesii* in an anaerobic digester, and by Miyagi *et al* (1995) with *Ruminococcus albus* introduced into goat rumen. *S. jonesii* rose to a level of ~1% of total rumen eubacterial rRNA after 10 days, but until then, had maintained itself at an order of magnitude lower. This bacterium took 10 days to establish itself at a level at which it could successfully detoxify 3,4 DHP, the toxic principle of *Leucaena,* even though this substrate was provided throughout the experiment. Miyagi *et al* (1995), using *R. albus* in goat rumens, showed that in one experimental animal *R. albus* levels dropped to ~10^2/ml, and plateaued at that level for 6 days until the experiment was terminated. In the other animal, levels dropped to just over 10/ml, and rose erratically to ~10^2/ml. Levels varied between 10 and 10^2/ml to the termination of the experiment after 14 days. Results from AR29 in sheep G8 showed that numbers of this organism steadily declined over a period of 10 days, and did not re-establish at detectable levels until day 70. The results

from this project show that reports of bacterial strains failing to establish should possibly be treated with caution, especially when trials only extend for a few days.

The success of the bacterial inoculations performed during this project could have been due to several factors. First, none of the strains used had been serially subcultured over long periods. All strains were maintained as frozen stocks, and recultured from these stocks to maintain as far as possible the characteristics of wild strains. Second, culture was in rumen fluid medium, based on clarified rumen fluid with added nutrients. Attwood *et al* (1988) had, in contrast, used a laboratory strain of *B. ruminocola*, B_14, grown on brain heart infusion broth. Third, relatively small inocula were used, 200 ml of stationary stage culture containing 10^8 -10^9 cfu/ml for cattle, and 50 ml of similar cultures for sheep. There was some concern that the use of very large inocula, which would rapidly produce very large numbers of cells in the gut below the rumen with the normal process of rumen efflux, might have some immunogenic effect, and produce antibodies to the introduced strains. This theory was untested, however successful immunisation of ruminants against methanogenic Archaea was demonstrated by West (1995) using inoculation of bacteria into the rumen. Under natural conditions, bacteria colonise the rumen from very small inocula, and readily pass from animal to animal, probably in the saliva. Therefore the use of large volumes of inoculant should not be necessary.

6.5. Implications of Tracking and Quantitation Results for Rumen Biotechnology.

A major aim of rumen biotechnology is the improvement of rumen function by the introduction of genetically manipulated organisms (GMOs) (Chapter 1, Section 1.4.). In order to have a useful effect, these GMOs must be present at a level within the rumen that will enable them to affect rumen function. McSweeney *et al* (1993) showed that it was necessary for *S. jonesii* to be present at a level of ~1% of eubacterial rRNA before it significantly detoxified 2,4 DHP. Gregg *et al* (1994) calculated that *B. fibrisolvens* OB156/pBHermF would need to be present at 0.5 - 1% of microbial protein to successfully detoxify fluoroacetate in the rumen.

Results obtained from the introduction of bacterial strains into the rumen in this project, and corroborated by the work of Miyagi *et al* (1995) showed that these levels may not be readily attainable. While it was shown to be possible to manipulate population

levels of different strains of bacteria by altering feed intakes (Chapter 3, p 50) levels of 1% of total rumen bacteria were not achieved by any strain.

In order to overcome this problem, Gregg *et al* (1993) suggested it might be possible to modify several strains. This would not only achieve higher levels of the introduced genetic material, but possibly overcome the problems of random fluctuations in populations of single strains that were apparent not only in this project, but in the results of Gregg *et al* (1993) on the introduction of *B. fibrisolvens* E14 into sheep rumens. Another approach (Wallace, 1994) would be to incorporate into the GMO some modification that gave it selective advantage within the rumen ecosystem. Wallace (1994) suggested that cellulase degrading capacity at a lower pH than that of indigenous rumen microflora might be one such advantage. An efficient lignin degrading capacity might be another. A further option is the identification of strains of rumen bacteria that may be naturally present at the required levels, and the genetic modification of these strains.

6.6. Estimation of Genome Size by PCR.

The size of the genome of any bacterial species for which there exists defined PCR target sequence whose copy number on the genome is known, can be estimated using the accurate quantitation system provided by competitive PCR. The technique compares favourably with the pulsed field electrophoresis of genomic DNA digested by restriction enzymes (e.g. Frutos *et al*, 1989; Bourgeois *et al*, 1989.) for the determination of bacterial genome sizes. Unlike the pulsed field electrophoresis techniques, PCR does not require the use of intact genomic DNA, so the preparation of template material is very much simpler. Results obtained (Chapter 4, p 4-46) for *P. ruminicola* AR20 and *B. fibrisolvens* AR10, 3.47 Mb and 1.52 Mb respectively were comparable with published genome size estimations for other bacterial species. Bourgeois *et al* (1989) gave genome sizes of between 1.75 Mb and 2.5 Mb for *Lactobacillus* and *Streptococcus* species; Stanier *et al* (1987) gave genome sizes of 3.84 Mb for *E. coli* and 1.65 Mb for *Desulfovibrio* sp..

6.7. 16S rDNA Restriction Fragment Length Polymorphisms.

This work was started because it was thought that shuttle plasmids were more likely to transform closely related bacterial strains than those that were only slightly related. Therefore a quick simple test which was capable of grouping bacterial strains into genetically similar clusters was considered desirable. The preliminary work on 16S rDNA restriction fragment polymorphisms (RFLPSs) suggested that this might be possible, but that digestion with several enzymes would be required. Moyer *et al* (1994) used a double digest with two four base restriction endonucleases for investigating relationships among bacteria from a hydrothermal vent.

As work on transformation of rumen bacteria progressed, it became apparent that close genetic relationship was not necessarily a useful indicator of transformation success. *B. fibrisolvens* AR14 was successfully transformed with the dehalogenase containing shuttle plasmid, pBHermF, developed for *B. fibrisolvens* OB156. 16S rDNA RFLPs suggested that the two strains are not closely related. Also the shuttle plasmid pBA, developed for *P.ruminicola* AR20, was successfully transformed into *P.ruminicola* AR29 (D. Schaefer and C. Cooper, pers. comm.). Genomic digests (Gregg and Ware, 1990) showed that these two strains were not closely related.

In contrast, the two *B. fibrisolvens* strains, AR10 and AR12 were genetically very close. This was shown by 16S rDNA RFLPs, DNA hybridisation studies (Hudman and Gregg, 1989) and genomic DNA restriction digests (Gregg, unpublished, pers. comm.). However, AR10 carried a cryptic plasmid that was not present in AR12. It had not proved possible to transform AR12 with this plasmid, or with any shuttle vector derived from it (Gregg and Beard (Ware), pers. comm.).

Because of the lack of correlation between genetic relationship and transformation success, no further work was carried out on 16S RFLPs of rumen bacteria.

6.8. Application of PCR Techniques to General Microbial Ecology.

While the PCR techniques of this project were developed specifically for rumen bacteria, they can be readily extended to all fields of microbial ecology. PCR is already widely used for tracking and quantifying microorganisms in the environment. Knowledge of microbial diversity and interactions is essential to the understanding of such diverse systems as soil nutrient cycles, sewage sludge digestion and human colonic functions. The

ability to detect specific microorganism in the environment, including released GMOs, pathogenic bacteria in soils or water supplies or new strains of commercially useful bacteria such as *Lactobacillus*, provides improvements in public health and safety as well as economic benefits. PCR techniques for tracking and quantitation, such as those developed in this project can provide advances in all these areas.