CHAPTER 3.

TRACKING RUMEN BACTERIA BY PCR.

PART 1. INTRODUCTION.

Summary.

This chapter describes the selection of DNA sequences for use as PCR probes to detect specific rumen bacteria; the testing of these probes for specificity and sensitivity, and the purposes for which the probes have been used in experimental investigations to detect and monitor specific bacteria in *in vivo* rumen experiments. Essential components of the techniques are the selection of rumen samples and the preparation of template material for PCR amplification.

Background to PCR Tracking Experiments.

At the beginning of this project, two strains of rumen bacteria were under particular consideration as targets for genetic manipulation within our laboratory. These were *Butyrivibrio fibrisolvens,* strain AR10, and *Prevotella ruminicola*, strainAR20. Both these strains had been isolated from sheep rumen contents in Armidale by Dr. Frank Hudman (Hudman and Gregg, 1989).

Later in the project, a second *B. fibrisolvens* strain, OB156, isolated from white tailed deer by Dr Bob Forster of CFAR, Ottowa, Canada, became the subject of genetic modification. Dr Cheryl Beard of the Institute of Biotechnology, University of New England, had succeeded in constructing a shuttle plasmid, pBHerm, which could successfully transform both *E. coli* and *B. fibrisolvens* OB156. Into this plasmid was cloned a fluoroacetate dehalogenase gene from *Moraxella species* (Kawasaki *et al,* 1984), and this construct, pBHf, was re-introduced into *B. fibrisolvens* OB156 without loss of *in vitro* dehalogenase activity (Gregg et al, 1990). A shuttle plasmid, pBA, had been developed for *P. ruminicola* AR 20, and work was proceeding on attempting to clone this into construct into another Armidale *P. ruminicola* strain, AR29 (Klieve et al, unpublished).

P. ruminicola had originally been classified as *Bacteroides ruminicola*, but were recently re-classified, as they appeared to have greater resemblance to the genus *Prevotella* than to the genus *Bacteroides*. (Shah and Collins, 1990). However, work in progress by Dr Linda Blackall at the Queensland University of Technology appears to suggest that *P. ruminicola* AR20 at least may belong more properly with *Bacteroides*. The question of correct classification of many rumen *Bacteroides/Prevotella* species (as well as that of practically all rumen bacterial species) remains open, and awaits full analysis of the genetic material of these organisms. However, for the purposes of this thesis, these bacteria will be classified as *Prevotella*, except when referring to published material, where the author's nomenclature will be followed.

Detection methods were required for all these four strains of bacteria, because, if genetic modification of these strains succeeded, they could not be released without a specific method for monitoring them after release. Moreover, before releasing genetically altered strains of rumen bacteria, it was considered desirable to find out whether the same strains occurred naturally within the rumen of the recipient animal, and to have some idea of population range within the rumen. This would allow estimation of the effect of genetic modification on the environmental fitness of the organisms.

Further considerations were to find out if the same strains of bacteria existed in different species of ruminants, whether bacteria could be re-introduced successfully from laboratory cultures to the rumen, and whether bacteria from one ruminant species could be successfully introduced into the rumens of other species. Experimental work in this area had been previously attempted by Attwood *et al*, (1988), Flint *et al*, (1989) and Gregg *et al*, (1993). See Chapter 1, section 1.5.

Attwood *et al*, (1988) introduced a laboratory strain of *P. ruminicola*, B_14, into the rumen of sheep. The organism was tracked by DNA hybridisation of total rumen DNA with a gene specific probe, and it was found that the numbers declined rapidly, and could not be detected after three hours, and remained undetectable at 48 hours after inoculation, having fallen to a level of less than 10^7 /ml of rumen contents, the lowest limit of detection by their methods. They suggested that this could be due to a strain specific bacteriocin, and that laboratory passaged bacteria might be difficult to establish in the rumen.

Gregg *et al*, (1993) used *B. fibrisolvens* strain E14, isolated from the rumen of reindeer by Dr Colin Orpin of CSIRO Division of Tropical Crops and Pastures, and genetically modified by Dr John Brooker to express tetracycline resistance, using the transposon Tn916. This was introduced into the rumens of four sheep and survival monitored by plating aliquots of rumen contents on tetracycline plates, and performing colony hybridisation at high stringency using E14 genomic DNA as a strain specific probe. These bacteria were monitored in the rumen for 50 days giving counts averaged over the four animals of between about 10^6 and 10^7/ml of rumen sample. Levels in individual animals varied between 2 x 10^7/ml to levels below the detection limit of ~10^4/ml, varying between animals over time. This study discounted the hypothesis of Attwood *et al*, (above), that laboratory passaged strains of rumen bacteria were unlikely to survive when re-introduced into the rumen. However, it was laboratory practice at the Institute of Biotechnology to maintain frozen stocks of rumen bacterial isolates, and keep passaging to a minimum by returning to these original isolates for fresh culture materials for experimental purposes. This was to maintain as far as possible the characteristics of the strain that had enabled it to survive under the highly competitive conditions of the rumen. The study (Gregg *et al*, 1993) also showed that rumen bacteria could be transferred from one ruminant species to another, at least in some cases.

It was against this background that work commenced on PCR detection methods for the specific rumen strains *B. fibrisolvens* AR10 and OB156, and *P. ruminicola* AR20 and AR29.

PART 2. EXPERIMENTAL WORK.

3.2.1. SPECIFICITY AND SENSITIVITY OF PCR PROBES.

A pre-condition for any PCR reaction is the synthesis of oligonucleotide primers complementary to the ends of the sequence to be amplified. Therefore some genomic DNA sequence of the organism of interest must be, or have been, determined. When primers have been chosen, and PCR amplification of the DNA sequence shown to be successful, it is important to know whether the sequence amplified is unique to the chosen organism, or can be detected in a number of different organisms. It is also desirable to know at what level the organism can be detected.

3.2.1.(A). Selection and Preparation of Target Sequences.

(i). Target Sequence for *Prevotella ruminicola* AR20.

The target sequence for *P. ruminicola* AR20 was part of the cel A gene of AR20, cloned into pUC 18 as the plasmid pJW4 (Woods *et al*, 1989) and sequenced by P. Vercoe (Vercoe and Gregg, 1995). This gene had been shown (Woods *et al*, 1989) to exist as only one copy on the *P. ruminicola* AR20 genome. The section chosen as the target sequence was bounded by the primers MTP1A and PVP2ex. (See appendix 1). The primers GA3I and G2D2 were synthesised complementary to sequence within the section bounded by MTP1A and PVP2ex, to give a shorter extension product (See Appendix 1).

(ii). Target Sequence for *Butyrivibrio fibrisolvens* AR10.

The target sequence for *B. fibrisolvens* strain AR10, was taken from the plasmid pDS49, which was derived from the plasmid pHS4, a pUC 18 plasmid with a dehalogenase gene from *Moraxella species* cloned into it. pDS49 was one of a series of plasmids with fragments of *B. fibrisolvens* AR10 genomic DNA cloned into the *Hind* III site of the pUC multiple cloning site to investigate their activities as gene promoters. The *B. fibrisolvens* AR10 pDS49 fragment had been sequenced (see Appendix 1), so it provided a possible PCR target sequence for AR10. The primers DS49top and DS49PE (see Appendix 1) had been synthesised to be complementary to sequences near each end of the 362 base pair genomic fragment.

(iii). Target Sequences for *B. fibrisolvens* OB156 and *P. ruminicola* AR29.

Because no DNA sequence was available for these two strains, genomic DNA had to be cloned and sequenced to provide specific primer sites.

Materials and Methods.

Partial digests of 3 aliquots of approximately 0.5 mg of genomic DNA from each organism were carried out with the 4 base restriction enzyme, *Sau* 3aI, for 10, 15 and 20 min. The DNA was then ethanol precipitated at room temperature, redissolved in distilled water and electrophoresed on a 1% agarose preparative gel. The fragments of each digest between approximately 400 and 800 bp in length were excised from the gel, combined, and purified using the Gene Clean method. Aliquots of this DNA were ligated into the *Bam* H I site of pUC 18 using the Sure Clone (Pharmacia) kit. *E. coli* K803 was electrotransformed with approximately 1 ng of the ligated DNA, and transformants were selected on ampicillin plates.

15 colonies were chosen for each bacterial strain, and presence of, and approximate length of, inserts checked by PCR, using the standard pUC forward and reverse 24mer primers. 100 µl of overnight culture from each colony to be tested was pelleted by centrifugation, washed, and resuspended in 10 µl water. 2 µl of this bacterial suspension was used as PCR template.

PCR conditions:- Denature 95° C, 5 min for 5 cycles, then 1 min for 15 cycles;
Anneal 65° C, 1 min, 20 cycles;
Polymerise 72 ° C, 1 min, 20 cycles.

5 µl of each amplification reaction was electrophoresed on 1% agarose gel, and visualised with ethidium bromide.

The cloned plasmid pGOB 5, with a genomic insert of approx. 650 bp DNA was selected from the *B. fibrisolvens* OB156 genomic clones. Similarly, pGAR 11, with approx. 500 bp of insert DNA, was selected for *P. ruminicola* AR29. The inserts were partially sequenced, using the Prism fluorescent sequencing kit. About 300 bp readable sequence was obtained from each plasmid, and primer pairs GOBF4 and GOBI were

synthesised for *B. fibrisolvens* OB156 together with GARF and GARI2 for *P. ruminicola* AR29. (See Appendix 1 for primer sites and sequences.)

(iv). Target Sequence for *Ruminococcus albus* AR67.

This cellulolytic rumen species was not under consideration for genetic manipulation. However, there was some interest in its distribution as a contribution to improved knowledge of rumen ecology.

A probe for AR67 was selected from known sequence of the endoglucanase gene, TC1, which had been cloned into pUC 18 as the plasmid pTCCT (Ware *et al*, 1989), and sequenced (Vercoe and Gregg, 1995). The target segment was defined by the 20 base sequencing primers PARR3 and PRA6 at positions 855 and 1349 on the gene sequence.

3.2.1.(B). Specificity of PCR Probes.

Materials and Methods.

PCR conditions.

Conditions for PCR amplifications of the target sequences are summarised in Table 3.2.1. Cycle numbers and amounts of template DNA are shown under individual reactions.

Table 3.2.1.

PCR Conditions for Detecting Specific Rumen Bacterial Strains.

Template	Primers	Denaturation Time	Annealing Temperature	Annealing Time	Extension Time
pJW4, *P. Ruminicola* AR20	MTP1A,PVP2X	90 sec	70 -72^{O}C	60 sec	60 sec
pJW4, *P. ruminicola* AR20	GA3I, G2D2	60 sec	65^{O}C	40 sec	40 sec
pDS49, *B. fibrisolvens* AR10	DS49top,DS49E	90 sec	70 -72^{O}C	60 sec	60 sec
pTCCT, *R. albus* AR67	PARR3, PRA6	90 sec	61^{O}C	60 sec	60 sec
pGOB5, *B. fibrisolvens* OB156	GOBF4, GOBI	60 sec	65^{O}C	40 sec	40 sec
pGAR11, *P. ruminicola* AR29	GARF,GARI2	60 sec	65^{O}C	40 sec	40 sec

For genomic DNA, the first three denaturation steps were for three minutes, to ensure complete denaturation of the longer template. After this, conditions were as specified above for the primer pairs used.

When intact bacterial cells were used as a source of template DNA, the first three denaturation steps were for 5 min to give time for the hot detergent solution of the PCR

buffer to degrade the bacterial cell membranes, allowing primers and enzyme access to the chromosomal DNA.

Each set of reactions included a negative control consisting of all PCR reagents without DNA.

After amplification, unless otherwise specified, 3 µl of loading buffer was added to each tube, and 7 µl of the resultant mixture was loaded onto 1% agarose gel for electrophoresis.

(i). Specificity of the Chosen PCR Probes.

Genomic DNA from a range of rumen bacteria was tested for the production of specific PCR amplification products, using the primers developed for *B. fibrisolvens* AR10 andOB156, and *P. ruminicola* AR20 and AR29. The bacterial strains tested were:

AR3 *Streptococcus bovis*
AR7 *Prevotella ruminicola*
AR9 *Butyrivibrio fibrisolvens*
AR10 *Butyrivibrio fibrisolvens*
AR12 *Butyrivibrio fibrisolvens*
AR14 *Butyrivibrio fibrisolvens*
AR15 *Butyrivibrio fibrisolvens*
AR16 *Butyrivibrio fibrisolvens*
AR20 *Prevotella ruminicola*
AR22 *Prevotella ruminicola*
AR23 *Prevotella ruminicola*
AR24 *Prevotella ruminicola*
AR27 *Butyrivibrio fibrisolvens*
AR29 *Prevotella ruminicola*
AR30 *Prevotella ruminicola a*
AR31 *Prevotella ruminicola*
AR33 *Prevotella ruminicola*
AR36 *Streptococcus intermedia*
AR53 *Selenomonas ruminantium*
AR67 *Ruminococcus albus*

Approximately 50 ng of genomic DNA from each strain was used as template, and amplified for 25 cycles, using the primer pairs MTP1A/PVP2ex, and GA1B/G2D2 for *P. ruminicola* AR20, and DS49top/DS49PE for *B. fibrisolvens* AR10.

The *B. fibrisolvens* OB156 and *P. ruminicola* AR29 primer pairs were tested against the same bacterial strains as *B. fibrisolvens* AR10 and *P. ruminicola* AR20, with the addition of *B. fibrisolvens* OB156, using the primer pairs shown in Table.3.2.1.

The *R. albus* AR67 primer pair was tested against AR67, *B. fibrisolvens* AR10 and *P. ruminicola* AR20 DNA. This was sufficient to show that the primer pairs for this species did not detect the strains of bacteria of major interest to this project. As the AR67

primer pair was taken from the endogluconase gene, it was assumed that it was likely to be specific for that gene in AR67, or genetically similar strains. This probe was a last minute addition to the experimental work, and as such was not tested against the full range of bacterial strains available.

Results.

(i) Specificity of Chosen PCR Probes.

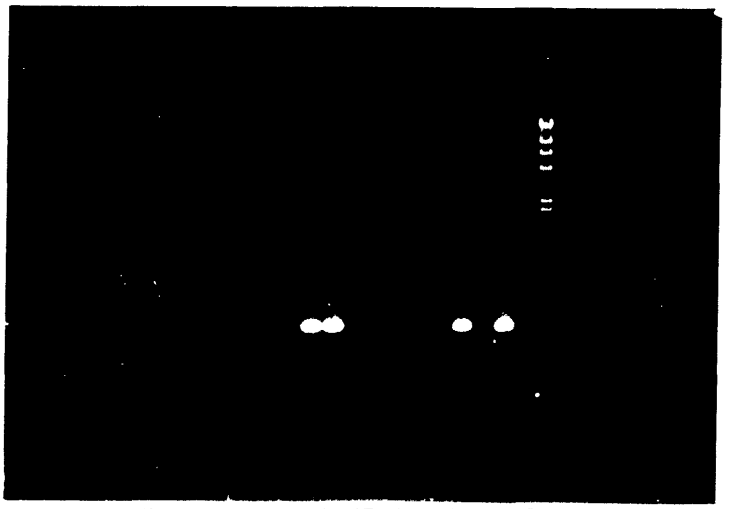
The *B. fibrisolvens* AR10 specific primers produced amplification product with *B. fibrisolvens* strains AR10 and AR12 genomic DNA only. *B. fibrisolvens* strains AR10 and AR12 were known to give essentially identical restriction patterns when their genomic DNA was digested with *Eco* R1, showing that their genotypes must be very similar, (Hudman and Gregg, 1989). Other species of *B. fibrisolvens* tested did not show the same restriction pattern, and did not produce specific amplification product with the *B. fibrisolvens* AR10 probe.

Both pairs of *P. ruminicola* AR20 specific primers produced identical amplification product with *P. ruminicola* strains AR7 AR20, AR22 and AR23 (data not shown). These strains of *P. ruminicola* shared a common restriction pattern not matched by any other of the tested strains of the same bacterial species. Thus they were taken to be sufficiently similar to be regarded as the same organism. It was therefore concluded that the *B. fibrisolvens* AR10 and *P. ruminicola* AR20 probes were specific for the DNA of the target organisms for which they were designed, or those of very closely related strains.

P. ruminicola AR29 primer pairs produced specific amplification product only with *P. ruminicola* strains AR29 and AR30 genomic DNA, although small amounts of non-specific products were amplified from the AR31 template. *P. ruminicola* AR29 and AR30 give practically identical genomic restriction patterns. Figure 3.2.1 illustrates the

Figure 3.2.1

Specificity of *P. ruminicola* AR 29 Primers



1 3 5 7 9 11 13 15 17 19 21 23 25
2 4 6 8 10 12 14 16 18 20 22 24 M

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	*AR29
4	#AR10	10	*AR22	16	*AR31	22	#OB156
5	#AR12	11	*AR23	17	*AR33	23	pGAR11
6	#AR14	12	*AR24	18	♣AR36	24	pGOB5

• *Streptococcus bovis*
* *B. fibrisolvens*
\# *P. ruminicola*
♣ *Streptococcus intermedia*
♦ *Selenomonas ruminantium*
♠ *Ruminococcus albus*

Lanes 14,15,21,23 show positive amounts of amplification product, corresponding in length to that from *P. ruminicola* AR29, and its target plasmid pGAR11
Small amounts of specific amplification products in the other lanes are false positives due to slight contamination in the reaction mix.
AR31 produced small amounts of a series of amplification products, all dissimilar to those produced by *P. ruminicola* strainsAR29 and AR30
Lane 25 shows λ/H*ind* III size marker.

amplification products from specificity testing of *P. ruminicola* AR29. It demonstrates that bacterial DNA, which gives rise to specific amplification products, can be clearly distinguished from that which does not. This photograph also illustrates the production of non-specific amplification products from amplification of *P. Ruminicola* AR31 using the *P. ruminicola* AR29 specific primer pair. Also shown is the appearance of false positive results, which demonstrate reagent contamination

B. fibrisolvens OB156 primer pairs (data not shown) produced amplification product only with *B. fibrisolvens* OB156 genomic DNA.

The *R. albus* AR67 specific probe (data not shown) showed no amplification product with either *B. fibrisolvens* AR10 or *B. fibrisolvens* AR20, but produced amplification product with *R. albus* AR67 genomic DNA.

3.2.1.(C). Sensitivity of PCR Probes.

Materials and Methods.

(i). Sensitivity of PCR Probes Using Plasmid and Genomic DNA.

The chosen primer pairs were used to amplify varying amounts of template DNA either from plasmids carrying the specific target sequences, or from the genomic DNA of target bacteria. Amounts of template DNA used, and the number of amplification cycles performed is summarised in Tables 3.2.2. and 3.2.3 below. Reactions were performed in duplicate for each amount of template shown in column 2. Plasmid DNA was amplified for both 30 cycles and 40 cycles, genomic DNA for 40 cycles. Finally, 1.0pg of *P. ruminicola* genomic DNA was amplified in the presence of varying amounts of "foreign" DNA to determine whether small amounts of genomic DNA could be detected against a background of non-specific DNA.

Table 3.2.2.

Template DNA and Cycle Number for Sensitivity of AR10 and AR20 Primer Pairs.

DNA Source	Amount of DNA//Reaction	No. of Cycles
pDS49	10.0, 1.0, 0.1, 0.01, 0.001 pg	30 and 40
B. fibrisolvens AR10	1000, 100, 10, 1.0, 0.1pg	40
PJW4	25, 2.5, 0.25, 0.025 fg	30 and 40
P. ruminicola AR20	10, 5, 1.0, 0.5, 0.1, 0.05 pg	40
P. ruminicola AR20 + *B. fibrisolvens* AR10	1.0 pg 1.0, 0.1, 0.01, mg	40

(ii). Sensitivity of PCR Probes Using Whole Bacteria.

a) Target bacteria diluted in water.

Stationary phase cultures of *B. fibrisolvens* AR10, *P. ruminicola* AR20 and *R. Albus* AR67 were centrifuged to pellet the cells. The cells were washed, by centrifuging and resuspending the pellet in water, twice, then the cells were pelleted by centrifuging. This pellet was resuspended in a volume of water equal to the initial volume of culture used. Tenfold serial dilutions of this suspension were made in water, and 2.0 µl of each of the 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions were used as template for 40 cycles of PCR amplification.

b) Comparison of target bacteria diluted in rumen sample and water.

The rumen sample was taken from a fistulated animal house sheep. The sample comprised rumen contents withdrawn from below the "raft" of plant material and strained through stretched nylon-stocking material, to eliminate large feed fragments while retaining material less than approximately 0.5 mm in diameter.

B. fibrisolvens OB156 bacteria were selected for this experiment as they could be more readily enumerated in a counting chamber than *P. ruminicola* or *B. fibrisolvens* AR10 cells. These strains did not always separate after cell division, which made it difficult to estimate cell numbers visually.

OB156 cells were enumerated in a counting chamber, and dilutions made to give approximately 77, 8, and 2 cells/5 µl. Dilutions were made in both water, and a sample of rumen contents known to contain no *B. fibrisolvens* OB156.

The rumen sample was heated in boiling water for 20 min, immediately after collection to destroy any DNAses present. Tenfold serial dilutions of the bacterial culture were made using the rumen sample as diluent, with a final fourfold dilution to give approximately 2 cells/ml. The dilutions were centrifuged, and the pellet was washed twice in 10 mM EDTA, and finally suspended in 5 x original volume of distilled water. 5 µl aliquots were used for PCR template (equivalent to 1 µl of "doped" rumen sample). The final fivefold dilution was to provide larger template volumes to improve reproducibility of results, as percentage errors in sample volume are lower with larger volumes. Duplicates of both water and rumen

sample dilutions were amplified for 35 cycles of PCR. After amplification, 3 µl of loading buffer was added to each tube, and 10 µl aliquots of each amplification were run on 2% agarose gel. The gel was photographed, and the negative scanned with a laser densitometer.

Results

(i). Sensitivity of Chosen PCR Probes Using Plasmid and Genomic DNA.

a) *P. ruminicola* AR20 Probe

pJW4 plasmid DNA was detected at levels down to 0.25 fg after 30 cycles, and 0.025 fg, i.e. approx. 4-5 copies, after 40 cycles.

P. ruminicola AR20 genomic DNA was detected at levels down to 0.05 pg after 40 cycles of PCR, which was the lowest level tested.

P. ruminicola AR20 genomic DNA was detected at a level of 1 pg in the presence of 1 mg of competing DNA, representing a DNA dilution of one part per million.

These results show very high sensitivity, with detection of the target sequence down to single figure copy numbers, and detection in the presence of a millionfold excess of competing non-target DNA.

b) *B. fibrisolvens* AR10 Probe.

pDS49 plasmid DNA was detected at levels down to 1 fg after 40 cycles of PCR.

B. fibrisolvens AR10 genomic DNA was detected at levels down to 1 pg after 40 cycles of PCR.

These results were less sensitive than the *P. ruminicola* AR20 probe results, possibly because of secondary structure in the *B. fibrisolvens* AR10 target sequence (see discussion). However, the detection limit of about a picogram of genomic DNA in a 20 µl

PCR reaction was considered adequate for the purpose of determining the presence of AR10 as a significant component of ruminal bacterial populations.

(ii). Sensitivity of PCR Probes Using Whole Bacteria.

a). Bacteria Diluted in Water.

2.0 µl *B. fibrisolvens* AR10, *P. ruminicola* AR20, and *R. albus* AR67 all produced detectable levels of specific amplification product after stationary cultures of these bacteria had been diluted to 10^{-5}. Dilutions of greater than 10^{-5} were not tested. Plate counts gave bacterial numbers from ~10^8/ml to 5 x 10^8/ml, so 2 µl of 10^{-5} dilution was expected to contain around 2-10 target cells. Results obtained later suggested that this number was an underestimate (see Chapter 5, section 2.6).

b) Bacteria diluted in Rumen Sample.

Results of the densitometer scans are shown in Table 3.2.2. below. They demonstrate that the presence of the rumen material inhibited PCR amplification.

Table 3.2.3

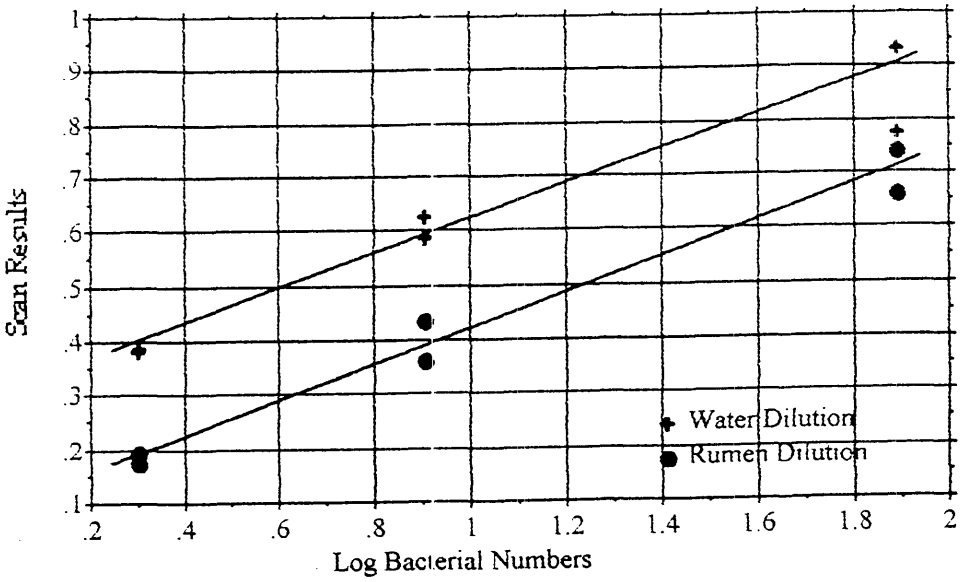
Densitometer Scan of PCR Amplification of Bacterial Dilutions.

Bacterial Nos.	Log Bacterial Nos.	Scan Readings Water Dilution.	Scan Readings Rumen Dilution.
2	0.301	0.375	0.172
2	0.301	0.384	0.194
8	0.903	0.627	0.362
8	0.903	0.586	0.432
77	1.88	0.933	0.738
77	1.88	0.775	0..660

When these figures were plotted graphically (see Graph 3.2.1. below), two results became apparent. First, there was a linear relationship between the logarithm of the numbers of bacteria used as PCR template and the amount of DNA produced as shown by the densitometer scan. This relationship appeared valid for both sets of dilutions. Secondly, the slope of both sets of scan results was virtually the same, but the rumen-diluted samples gave a densitometer reading equivalent to water-diluted samples containing approximately one quarter the number of bacteria.

Graph 3.2.1.

Comparison of PCR of Whole Bacteria in Water or Rumen Dilutions.



The use of whole cells as PCR template meant that template preparation could be considerably simplified. This saved time, effort, and reagents, and also reduced the chance of accidental contamination of specimens. However, template volume was limited to a sample size of 1 µl of rumen sample per 20 µl of PCR reaction.

However it was found, when Bresatec altered the composition of its buffer, that a PCR buffer with a high detergent content is required for the use of whole bacteria as PCR template material. The Bresatec buffer used provided a Triton X-100 concentration of

0.45% in the reaction mixture, and it was found possible, although unnecessary, to use Triton X-100 concentrations of up to 0.6% without affecting the sensitivity of the reaction.

The presence of washed rumen material inhibited the PCR reaction. This inhibitory effect of rumen material was seen in all PCR amplifications performed in the presence of such material. (results not shown). However it was shown to be possible to detect as few as two bacterial cells/µl of rumen sample in the case of *B. fibrisolvens* OB156. Since rumen bacterial counts are estimated to be of the order of 10^{10}/ml, this particular bacterium could be detected when it comprised less than one millionth of the rumen bacterial population.

3.2.2. PREPARATION OF RUMEN SAMPLES FOR PCR.

There were two choices of template materials for PCR amplification of specific bacteria in rumen samples, extracted DNA or whole cells, both of which had been shown to provide effective templates for PCR amplification. However it was necessary to determine whether any component of rumen samples would seriously inhibit the PCR reaction.

Because it was known that fibrolytic rumen bacteria adhere strongly to plant material (Stewart and Bryant, 1988), small plant fragments were retained in rumen samples for PCR tests, and the adherence of *B. fibrisolvens* AR10 to such material was investigated.

Enzymic methods using lysozyme, based on the methods of Marmur (1983), were chosen for extraction of DNA from rumen samples, as it was considered that these methods would preferentially extract DNA from bacteria rather than from intact cells of plant material present in the sample.

Materials and Methods.

(i). Effect of rumen fluid on PCR Amplification.

0.1 ng of pJW4 plasmid DNA was amplified for 20 cycles in the presence of 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, and 5 µl of cell free rumen fluid.

(ii). Distribution of *B. fibrisolvens* AR10 between Plant Fragments and Liquid Phase in a Rumen Sample.

A preserved rumen sample, known to contain *B. fibrisolvens* AR10, which had been heated to 100°C after collection and kept at -70°C in the laboratory, was well shaken to mix the contents. A sample was taken through a Pasteur pipette, which allowed the inclusion of plant fragments up to approx. 1.0 mm in diameter. This was filtered through Watman No. 1 filter paper, which allowed bacteria to pass into the filtrate, but retained plant material. The filtrate was washed twice on the filter with water to ensure elution of the majority of those bacteria, which did not firmly adhere to plant fragments. The filtrate was centrifuged to pellet the bacteria, and the pellet resuspended in the

original sample volume of TE buffer, pH8. Plant material was also resuspended in the original sample volume of TE buffer, pH8.

The experiment was repeated using hot PCR buffer for washing the plant material. The rumen sample was pelleted by centrifugation, resuspended in hot (95°C) PCR buffer, and vortexed briefly, before filtration and washing as above with hot PCR buffer.

2 µl of each filtrate and each lot of washed plant material were amplified separately for 35 cycles using primers DS49top and DS49PE under conditions specified in Table 3.2.1. After amplification, 5 µl of each reaction was electrophoresed on 2% agarose gel, and stained with ethidium bromide.

(iii). Enzymic Preparation of Bacterial DNA from Rumen Fluid.

Preparation methods were based on the protocol for preparing DNA from cultured rumen bacteria described in the materials and methods section (Ch. 2, page 9).

750 µl samples of rumen material (collected as described in 3.2.1.c.) were diluted to 1.5 ml with TE buffer to reduce viscosity. Samples were centrifuged to pellet solid material, and the pellet was washed twice with TE buffer, by resuspension and centrifugation. Samples of late log phase or stationary phase cultures of the rumen bacteria *R. albus* AR67, *P. ruminicola* AR20, *B. fibrisolvens* AR10, *Streptococcus bovis* AR3, *Streptococcus intermedia* AR36, and *Selenomonas ruminantium* AR53 were tested in the same manner as the rumen fluid samples to check that the preparation methods worked equally well on a range of rumen bacteria, a requirement if this method of preparation was to produce an unbiased sample of DNA.

Pellets were suspended in 300 µl lysozyme in TE buffer with concentrations varying from 5 mg/ml lysozyme to 30 mg/ml lysozyme, and incubated for times varying from 30 min to overnight, at 37°C.

After lysozyme incubation, 10 µl RNAse A, 10 mg/ml, was added to degrade RNA, and SDS was added to concentrations varying from 0.1% to 2%, with incubation at 37°C for 30 min.

10 - 40 µl Proteinase K, 20 mg/ml , was then added, and samples were incubated at 55°C for periods ranging from 1 hr to overnight. The higher temperature increased the efficacy of the SDS as a cell membrane denaturant, without adversely affecting the activity of the Proteinase K.

As a check on the overall efficiency of lysis, aliquots of the rumen sample lysates were diluted to original volume and compared microscopically with dilutions of the original samples to estimate the proportion of cells successfully lysed.

The lysed samples were then centrifuged to remove undissolved material, and the supernatant extracted with buffered phenol, phenol/chloroform and chloroform. DNA was precipitated with 2.5 volumes of ethanol, and dissolved in 50 µl water. 5 µl aliquots were electrophoresed on 1% agarose gels stained with ethidium bromide to compare amounts of DNA produced by each treatment.

Results.

(i). Effect of rumen fluid on PCR Amplification.

Rumen fluid could be added in amounts up to 1 µl/20 µl reaction volume (i.e. 5% v/v) before it had any detectable effect on the yield of amplification product.

This meant that rumen samples could simply be washed before amplification, as residual amounts of rumen fluid would not interfere with the reaction.

(ii). Distribution of *B. fibrisolvens* AR10 Cells Between Plant Fragments and Liquid Phase in Rumen Samples.

Figure 3.2.1 shows amplification products of the leaf and eluate samples. It can be seen that after washing with water, the greater part of the *B. fibrisolvens* AR10 amplification product derived from the plant material fraction rather than the water eluate, showing that the majority of AR10 cells were bound to the plant material. Washing in hot PCR buffer detached about half the AR10, but the rest remained bound. This showed that AR10 adhered firmly to the plant fragments. More importantly, it demonstrated that bacteria bound to plant fragments could act as template for PCR.

Figure 3.2.2.

PCR Amplifications of Plant Fragments and Eluted Bacteria.

Lane 1, negative control; Lane 2, λ/*Hind* III size marker;
Lane 4, whole sample; Lane 5, water eluate; Lane 6, water washed plant material;
Lane 7, PCR buffer eluate; Lane9 buffer washed plant material;
Lane 9, positive control; Lane 10 λ/*Hind* III size marker.

(iii). Enzymic Extraction of Bacteria DNA from Rumen Samples.

Optimum yields of DNA from rumen samples and from *R. albus* AR67 were obtained using the conditions listed below. Other bacterial strains yielded maximum DNA under lower concentrations of enzymes and SDS, but the use of higher concentrations did not lessen the yield from these strains This was therefore adopted as the method for routine extraction of DNA from rumen material.

a) Pelleted, washed material from each rumen sample was suspended in 0.04 x original sample volume of lysozyme (20 mg/ml in TE buffer) and incubated 90 min at 37°C.

b) RNAse A and 10% SDS were added to give concentrations in the sample of 10 mg/ml and 2% respectively. The sample was incubated at 37°C for a further 30 min.

c) Proteinase K was added to give a concentration of approx. 0.1 mg/ml, and the sample incubated at 55°C overnight.

d) Solvent extractions and precipitation were performed as for genomic DNA purification (See Materials and Methods Chapter 2, pp 9 -10).

Microscopic examination of the lysate produced by the above method after Proteinase K treatment showed that the lysate contained intact bacterial cells approximately equal in numbers to between 1/16 and 1/32 dilutions of the original sample. i.e. approximately 95% of rumen bacteria were lysed by this treatment. Surviving cells were of several forms, so it did not appear that any one bacterial species totally resisted lysis.

3.2.3. NORTH QUEENSLAND RUMEN SURVEY.

Materials and Methods.

(i). Collection and Preservation of Rumen Samples .

Rumen material had been collected from sheep, cattle and goats from a number of North-West Queensland properties by Helen Sharpe and Terry McCosker. These samples were heated in boiling water as soon as possible after collection, stored at -70°C as soon as they arrived at the laboratory, and kept at that temperature for about two years.

(ii). PCR Template Preparation.

Samples were shaken thoroughly to suspend solid material, and sampled using a Pasteur pipette with an aperture of approx. 1.0 mm diameter, so as to include small particles of the suspended plant material. 750 µl of rumen sample was diluted with an equal quantity of TE buffer, and centrifuged for 5 min. The supernatant was discarded, the pellet washed twice more in TE buffer, suspended in 300 µl lysozyme (20 mg/ml in TE buffer), and incubated at 37°C for 90 min. 60 µl 10% SDS and 2 µl RNAse A (10 mg/ml), were added, and mixed by inversion, and incubated at 37°C for a further 30 min. The mixture was then incubated with 20 µl Proteinase K (20 mg/ml), at 50-55°C overnight. The digest was centrifuged for 5 min, and the supernatant extracted twice with phenol/chloroform,1:1; and twice with chloroform. DNA was precipitated with 2.5 volumes of ethanol, or 0.6 volumes of propan-2-ol, and redissolved in 30 µl water. Excess discoloration due to contaminating material was removed when necessary by diluting the sample to 100 µl with water, and re-precipitating the DNA with ethanol.

2 µl of the DNA solution was used as PCR template, equivalent to DNA extracted from 50 µl of rumen sample. Seventy one specimens were tested for the presence of *B. fibrisolvens* AR10, *P. ruminicola* AR20, and *R. albus* AR67 in separate PCR reactions. Each set of reactions included a negative control consisting of all PCR reagents without DNA, and a positive control consisting of 0.01 ng of control plasmid DNA.

(iii). PCR conditions

As for genomic DNA conditions in section 3.2.1.(v)., and Table 3.2.1. Each sample was amplified for 30 cycles of PCR.

Results.

Full results of all tests performed are given in Table 3.2.5.. A summary of the results is given in Table 3.2.4..

Table 3.2.4.

Distribution of Bacterial Strains in North Queensland Ruminants.

	Cattle	Sheep	Goats
Total no. Sampled	57	8	6
No. Showing AR10	0	0	0
No. showingAR20	12	2	0
No. Showing AR67	2	1	0

From these Tables it can be seen that AR10, an Armidale strain of *B. fibrisolvens* isolated from sheep rumen, did not appear in any sample.

P. ruminicola AR20 and *R. albus* AR67, isolated from the same Armidale sheep as *B. fibrisolvens* AR10, were detected in both sheep and cattle, with AR20 being detected more frequently.

No goats showed the presence of any of the target bacterial strains, though the number tested was too small to draw absolute conclusions. ($p = 0.16$, assuming that the presence of *P. ruminicola* AR20 in any animal is independent of the presence of AR67.)

Table 3.2.5.

North Queensland Rumen Survey.

Station	Animal	No. Sampled	No. with AR10	No. with AR20	No with AR67
HEADINGLEY	Cattle	6	0	0	0
	Sheep	0	0	0	0
	Goats	0	0	0	0
YARROMERE	Cattle	5	0	2	1
	Sheep	0	0	0	0
	Goats	0	0	0	0
OAKLEY	Cattle	5	0	0	0
	Sheep	0	0	0	0
	Goats	0	0	0	0
OLD LINDA DOWNS	Cattle	4	0	1	0
	Sheep	3	0	1	0
	Goats	0	0	0	0
THE LAKES	Cattle	5	0	2	1
	Sheep	4	0	1	1
	Goats	4	0	0	0
SWANLEA	Cattle	5	0	3	0
	Sheep	0	0	0	0
	Goats	0	0	0	0
GREGORY SPRINGS	Cattle	6	0	0	0
	Sheep	0	0	0	0
	Goats	0	0	0	0
CORINDA	Cattle	5	0	1	0
	Sheep	0	0	0	0
	Goats	0	0	0	0
MIRTNA	Cattle	5	0	0	0
	Sheep	0	0	0	0
	Goats	0	0	0	0
STANBROKE	Cattle	5	0	2	0
	Sheep	0	0	0	0
	Goats	0	0	0	0
FORTUNA	Cattle	6	0	1	0
	Sheep	1	0	0	0
	Goats	2	0	0	0

There was no apparent geographic correlation between the properties where animals were shown to be carrying the target strains of rumen bacteria, and those where animals were not shown to carry those bacteria.

The fact that many samples tested negative to all of the target bacteria could not be taken as proof that these organisms were not present. The experiment was designed to detect bacteria present at a reasonably significant proportion of the rumen population than to detect very low levels of the target strains. The lower limit of detection was roughly estimated to be approximately $5 \times 10^3 - 5 \times 10^4$ cells/ml (data not shown).

3.2.4. HEADINGLEY *B. FIBRISOLVENS* EXPERIMENT.

B. fibrisolvens AR10 had not been found in any of the animals surveyed in the previous experiment. AR10 was one of the organisms under consideration for genetic modification. It was accordingly decided to find out if this organism, originally a sheep rumen strain from UNE Armidale, could be introduced into cattle in North West Queensland and could survive at significant levels.

This work was carried out with the cooperation of the manager of Headingley Station, Clayton Paige, and the DPI Veterinary Officer at Mount Isa, Barry Robinson.

Materials and Methods.

(i). *B. fibrisolvens* AR10 Cultures.

200 ml cultures of *B. fibrisolvens* AR10 were grown to stationary phase in the laboratory in RF medium, and kept on ice for two days during delivery to Headingley Station.

(ii). Experimental Animals.

A herd of 40 Santa Gertrudis cows, kept separate from other cattle on the station, and managed under field conditions, was used as the experimental group.

(iii). Inoculation of cultures.

200 ml of *B. fibrisolvens* AR10 culture was introduced into the rumens of 5 cows out of the herd of 40 via throat tube. Unfortunately, due to adverse conditions at the time of inoculation, monsoon rain, and uncooperative cattle, pre-inoculation rumen specimens were not obtained. The results of the previous experiment, however, suggested that AR10 was unlikely to be present in Headingley cattle. Neither sheep nor cattle sampled from any station had tested positive to AR10.

(iv). Collection of samples.

Rumen samples were obtained from the inoculated cattle, and others in the herd, at intervals, over a period of just over 12 months. Samples were collected in similar manner to those for the previous North Queensland experiment, allowing comparison between the two experiments. Samples were collected via nose tubes, as animals tended to bite

through throat tubes. After collection, samples were heated to 100°C in boiling water, and then placed on ice for delivery to Armidale. They were stored at -20°C at the laboratory until they were tested.

(v). Template preparation.

The material was sampled using a Pasteur pipette to ensure inclusion of fragmented plant material. 750 µl aliquots were diluted 1:1 with water, and centrifuged. The pellet was washed twice in water, by resuspension and centrifugation, and resuspended in 0.5 – 1.0 volumes of water, depending on the apparent amount of solid material in the pellet. 2 µl of this suspension was used as DNA template.

(vi). PCR conditions.

Amplification was for 35 cycles, with the first three denaturation steps extended to 5 min. Remaining PCR conditions were as shown in Table 3.2.1. for *B. fibrisolvens* AR10.

Results.

Results of tests on the 5 inoculated cows are summarised in Table 3.2.6. Summarised results for uninoculated cows are given in Table 3.2.7., and for the full herd in Table 3.2.8.. Results of all tests are shown in Table 3.2.9., and the information from the first three tables is graphically represented in Graph 3.2.2. Fig. 3.2.2. shows a photograph of the products of PCR amplification of a series of rumen samples electrophoresed on an agarose gel, and illustrates the DNA levels which correspond to results reported strongly positive and weakly positive.

While it cannot be definitely ascertained that *B. fibrisolvens* AR10 was not present in the herd prior to inoculation, the results obtained are consistent with what could be expected if a new bacterial strain was successfully introduced into a group of cattle. However, comments on the spread of bacteria through the herd should be read with the proviso that it remains possible that the AR10 strain, or one genetically similar, was present in the herd prior to inoculation.

It should be noted, however, that calves born to the herd were able to acquire this bacterial strain, most probably from their mothers.

(i). Inoculated cows.

Table 3.2.6

Presence of AR10 in Inoculated Cows.

Days Post Inoc.	7	14	28	70	126	268	404
No. of Cows Tested	5	5	4	5	5	5	5
No with AR10	4	1	2	5	1	1	3
% with AR10	80	20	50	100	20	20	60

From Table 3.2.6., and Graph 3.2.2. it can be seen that the numbers of inoculated cows testing positive to AR10 fluctuated markedly over the term of the experiment. Graph 3.2.2 in particular shows that the level of AR10 found within any one particular cow also fluctuated markedly over the term of the experiment.

(ii). Uninoculated Cows.

Table 3.2.7.

Presence of AR10 in Uninoculated Cows.

Days Post Inoc.	7	14	28	70	126	268	404
No. of Cows Tested	4	0	20	19	19	27	30
No with AR10	3	-	17	10	6	1	10
% with AR10	75	-	85	53	32	4	33

From Table 3.2.7. and Graph 3.2.2. it can be seen that AR10 appeared to spread rapidly from inoculated cows to the uninoculated members of the herd. Of four uninoculated cows tested 7 days after the date of inoculation, three tested positive to the presence of AR10. 28 days after the date of inoculation, 17 out of 20 cows tested

positive to the presence of AR10 . Table 3.2.9 shows that the fluctuations in levels of AR10 between and within cows, which were seen with inoculated cows, were also seen with the uninoculated cows.

(iii) Total Herd.

Table 3.2.8.

Presence of AR10 in All Cows.

Days Post Inoc.	7	14	28	70	126	268	404
No. of Cows Tested	9	5	24	24	24	32	35
No with AR10	7	1	19	15	7	2	13
% with AR10	78	20	79	60	29	6	37

From Table 3.2.8. and Graph 3.2.2. it can be seen that AR10 apparently spread from the 5 inoculated cows to the majority of cows in the herd within the first seven days after inoculation. The high proportion of cows testing positive to AR10 was maintained at the next sampling date, 28 days after inoculation, which was the date of the first large scale sampling of the herd. After this date, the proportion of cows with AR10 declined at an almost linear rate to the sampling date of 268 days after inoculation, at which only 2 out of 32 cows tested positive to AR10. However, the final sample, taken 404 days after inoculation, showed that the proportion of cows testing positive to AR10 had risen again to 37%.

Graph 3.2.2.

Persistence and Spread of AR10 in Headingly Cattle.

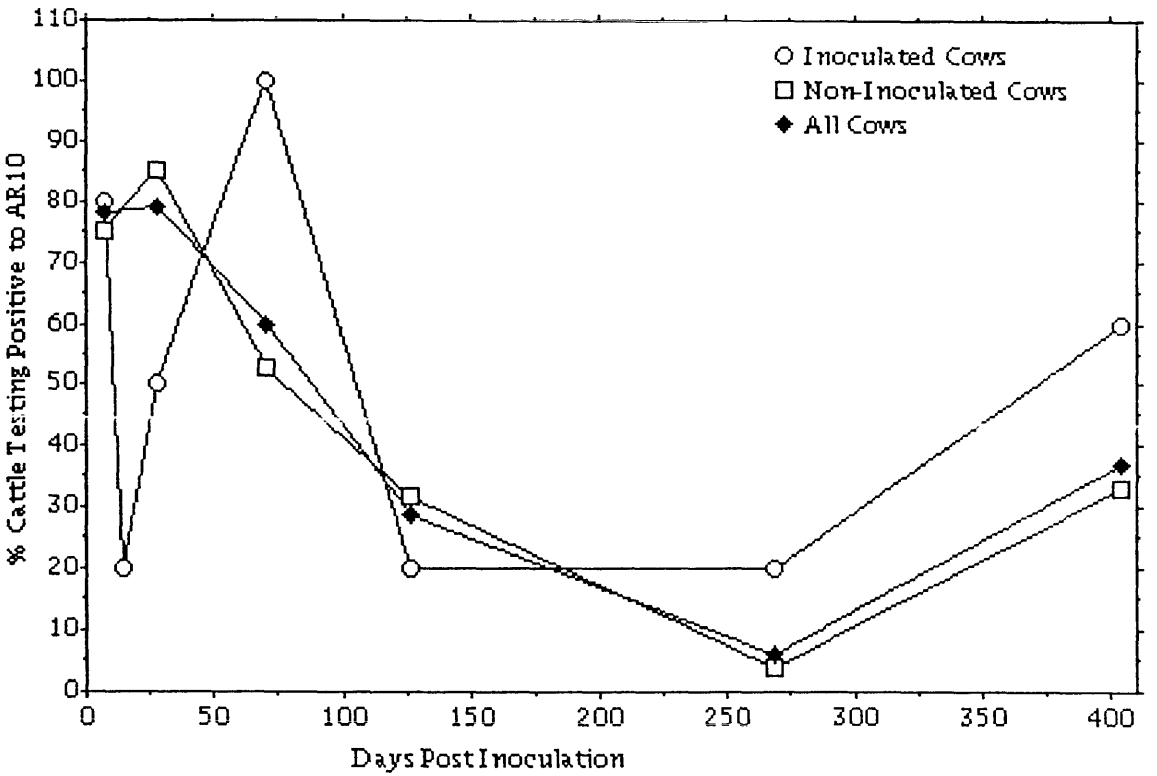


Table 3.2.9. Headingley *B Fibrisolvens* Experiment.

	Days Post Inoculation						
Animal No.	**7**	**14**	**28**	**70**	**126**	**268**	**404**
191	0	+++	+++	+++	0	0	+++
192	+	0	0	+++	0	0	0
193	+	0	0	+++	0	0	+
194	+	0	+	+	0	0	0
195	+++	+		+	+	+	+++
196			+		+	0	0
197				+	0	0	0
198			+	+	0	0	0
199	+		+		0	0	
200	+		+	+		0	
201	+++		0		+++	0	+
202			++	+++		0	+
203			+	0		0	0
204	0			0	0		+
205			+++	+++	0		0
206							0
207			+++	+++	0	0	
208			+++			0	+
209					0	0	0
210				0		0	0
211			0	0		0	0
212					0	0	0
213				0		0	
214			+++		+++		+
215				0	0	0	
216			+++	+			0
217					+++	0	0
218			+	+			+
219				+	0	0	
220			+		+		0
221			+		0	0	
222					0	0	
223				0		+++	
224			0	0		0	
225						0	
226			+++		0	0	0
227			+			0	0
228			+++		+++	0	
229				+++			0
230					0	0	+

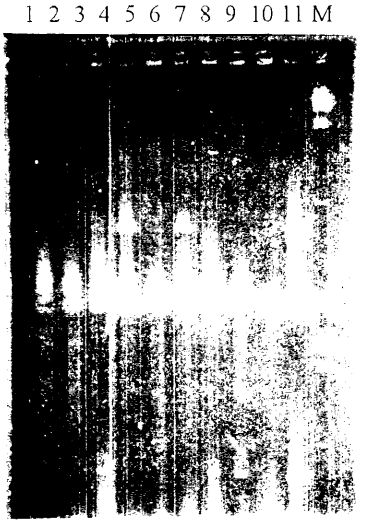
Italics identify inoculated animals

0 shows animals negative to AR10; + shows animals weakly positive to AR10; +++ shows animals strongly positive to AR10 (see figure 3.2.3)

Five calves born to the herd were tested on day 404, 3/5 tested at + level

Figure. 3.2.3.

Amplification Products of R ımen Samples from Headingley Cows.



Amplification products of rumen samples fr ɔm Headingley cows taken on day 404 of the experiment.
Lanes 4 & 6 show DNA levels reported as strongly positive to AR10.
Lanes 3,13,17,18,19 show DNA levels reported as weakly positive to AR10.
Lanes 10 & 20 are negative controls, and lanes 11 & 22 are amplifications of 0.01 ng pDS49 DNA used as positive controls.
Marker is λ/*Hind* III DNA.

3.2.5. PERSISTENCE OF, AND INFLUENCE OF DIET ON, TWO STRAINS OF *B. FIBRISOLVENS* AND TWO OF *P. RUMINICOLA* INTRODUCED INTO SHEEP RUMENS.

The Headingley experiment had shown that it was apparently possible to introduce strains of bacteria from the laboratory into animals in the field,. However, because no pre-inoculation tests of the animals had been possible, the results were not entirely unambiguous.

The Headingley results had also shown a possible dependence of levels of *B. fibrisolvens* AR10 on forage type, a not unexpected result in view of the fact that the rumen is a complex ecosystem with a wide variety of bacteria whose relative populations depend on nutritional intake. The use of PCR promised a way to follow particular genetic strains rather than overall species, and the opportunity to track different strains of the same species, and determine how dietary changes affected different strains.

At the time these experiments were contemplated, a genetically modified form of *B. fibrisolvens* OB156 had been produced, which had been transformed with the plasmid pBHermF, and which was able to detoxify fluoroacetate *in vitro*. Project members were concerned to find out whether this modified organism, which was originally isolated from the Canadian white tailed deer, could survive in sheep rumen, and whether the plasmid was stable under *in vivo* conditions.

Materials and Methods.

(i). Experimental Animals.

Two Dorset x Merino wethers, ear tagged G8 and G9, and with rumen fistulae were used. They were housed in metabolism crates, about 1.5 m apart, and unable to make physical contact with each other for the first 90 days of the experiment. They were then placed in a floor pen for three weeks to comply with Ethics Committee guidelines, and returned to metabolism crates for the remainder of the experiment. The animal house was GMAC approved at PC2 containment level, and was heated in winter to provide reasonably uniform temperature conditions over the course of the experiment.

Because genetically modified organisms were involved, strict precautions were observed. Since *B. fibrisolvens* OB156 is an obligate anaerobe, unable to tolerate even

microaerobic conditions, its chances of survival outside the rumen or anaerobic chambers was minimal. Nevertheless, protective clothing was worn in the animal house, all material coming from the animal house, except for rumen samples in sealed containers, was securely wrapped and disposed of by incineration, and the animal house was ventilated under negative pressure, with exhaust air being filtered to the outside atmosphere. At the end of the experiment, the animals were sacrificed, wrapped and incinerated.

(ii). Feeding.

The animals were automatically fed hourly throughout the twenty four hours, to reduce diurnal fluctuations in rumen conditions, and water was provided *ad lib*.

Diets were as follows:

500g oat chaff, 200g lucerne hay, standard maintenance diet, given up to day 19, and from days 72 -135

700g oat chaff, days 20 - 61

800g wheat straw, days 62 -71

600g lucerne hay, days 136 - 152.

Changes in diet were introduced gradually over 2-3 days to avoid digestive upsets

(iii) Bacterial Inocula.

Four bacterial strains were inoculated into each sheep: *B. fibrisolvens* strains AR10 and OB156, and *P. ruminicola* strainsAR20 and AR29.

Because of AQIS regulations, bacteriological peptone could not be used in any bacterial medium for inoculation into animals, as this material is imported from Europe, and there was concern that imported material made from meat might contain transmissible pathogens, such as BSE.

When the bacteria were tested in RF+ medium made with extra yeast extract to replace the peptone, it was discovered that *B. fibrisolvens* OB156 needed the addition of 1 mg/100 ml $CaCl_2$ in the medium, and *P. ruminicola* AR29 required the addition of 0.5 g/100 ml haemin. However it was later discovered that all four bacterial strains would grow in clarified rumen fluid to which was added approximately 1 g milled lucerne, or milled oat chaff per 100 ml, which would have been a simple and effective inoculation medium.

Bacteria were passaged three times in the yeast extract medium to ensure total elimination of peptone, and 50 ml of stationary phase culture of each bacterium was inoculated into the sheep via the rumen fistula.

Prior to inoculation, both sheep were tested for the presence in their rumens of all four bacterial strains. *B. fibrisolvens* AR10 was detected at low level in G9, however it was decided to inoculate this strain into both sheep, so that starting conditions would be similar for both of them.

(iv). Sampling Methods.

Rumen samples were removed via the rumen fistula. A hollow probe was inserted into the rumen below the level of the fibrous vegetable "raft", and samples were withdrawn into a 25 ml syringe. The end of the probe was covered in stretched nylon stocking material to exclude gross vegetable matter, but admit particulate material under approximately 0.5 mm in diameter, which could be expected to have many adherent bacteria. Sampling instruments were kept separate for each sheep, and carefully washed and disinfected between uses. Samples were taken twice weekly, except for the period from 90 to 130 days when sheep were not sampled. At the beginning of this period, one sheep developed an infection around its rumen fistula, and was treated with antibiotic (tetracycline)for two weeks It was considered that antibiotic treatment might affect rumen populations, and that it would be prudent to allow a two- three week recovery period before sampling was resumed..

(v). Sample Preparation.

After collection samples were immersed in boiling water for 30 min to destroy DNAses. All further sample preparation took place in a laminar flow cabinet to prevent contamination. Aliquots of 100 µl volume were diluted to 500 µl with sterile TE buffer and centrifuged to pellet the rumen material. The pellet was washed once more with TE buffer and once with sterile water before finally being suspended in 500 µl 10 mM EDTA. The presence of EDTA helped to prevent agglomeration of the rumen material during storage, however the concentration had to be kept low to avoid sequestration of significant amounts of magnesium in the PCR buffer during analysis. Washing was done in sterile 1.5 ml screwtop microfuge tubes, which were opened only in the laminar flow

cabinet. After washing and resuspending, the samples and their parent material were stored at -20^{0}C.

(vi). PCR Primers.

Because the *B. fibrisolvens* AR10 primer pair, DS49top and DS49PE were less sensitive than other primer pairs, other sequenced fragments of *B. fibrisolvens* AR10 genomic DNA were considered. These consisted of other DS plasmids, similar to pDS49, but with different AR10 genomic inserts. The plasmid pDS63 provided a sequence of genomic DNA with no internal secondary structure, and the primers DS63top and DS63PE defined a 282 bp target sequence. The primer pair was tested for selectivity as in 3.2.1.2.(ii) above, and proved specific to *B. fibrisolvens* AR10 and AR12. Sensitivity testing showed the primer pair sensitive enough to detect 0.1 fg of plasmid DNA after 35 cycles of PCR. Lower limits of detection were not tested.

To detect the presence of the dehalogenase gene, the sequencing primers CC5 and CC6.2I, defining a 300 bp sequence of the dehalogenase gene between bases 199 and 499, were used.

The primer pairs for *P. ruminicola* AR20 were GA3I and G2D2;
for *P. ruminicola* AR29 were GARF and GARI2;
for *B. fibrisolvens* OB156 were GOBF4 and GOBI

(vii). PCR Conditions.

Denaturation: 95^{o}C, 5 cycles of 5 min + 30 cycles at 1 min.

Annealing: 65^{o}C for 40 sec for all 35 cycles.

Polymerisation: 72^{o}C for 40 sec for all 35 cycles.

5 µl of prepared sample (equivalent to 1 µl of rumen contents) was used as template. Each set of reactions included a negative control, and a positive control with a known amount of template.

After all cycles had been completed, 3 µl loading buffer was added to each tube, and 7 µl of the mixture was loaded on to 1.5% agarose gel for electrophoresis

a) Fluctuations in bacterial numbers in response to dietary changes.

To detect fluctuations in bacterial numbers in response to changes in diet, 20 samples from each sheep were simultaneously subjected to PCR using each pair of primers in turn. Each set of forty samples had the reaction mix premixed and aliquotted into the reaction tubes to minimise inter-tube fluctuations in reaction conditions. Because amplification was performed on all samples at the same time and with the same reaction mix, samples could be compared within each amplification.

DNA templates for positive controls consisted of:

For *B. fibrisolvens* OB156: pGOB5 plasmid DNA at ~10^5 copies/ml

For *B. fibrisolvens* AR10: pDS63 plasmid DNA at ~10^6 copies/ml

For *P. ruminicola* AR20: pJW4 at ~10^6, 10^5, 10^4 copies/ml and

AR20 bacterial cells at ~10^6, 10^5, 10^4, 10^3 copies/ml

For *P. ruminicola* AR29: pGAR11 at ~10^5, 10^6, 10^7 copies/ml

Each gel was loaded with the amplified products of one primer pair. The G8 samples were loaded first, and electrophoresed for thirty minutes, then the G9 samples were loaded onto the same gel, thus ensuring that all the amplifications from each primer pair were equally stained, and comparable. The gels were stained with ethidium bromide, photographed, and the negatives scanned by laser densitometry.

b) Stability of the pBHermF plasmid in *B. fibrisolvens* OB156

To check for stability of the pBHermF plasmid in *B. fibrisolvens* OB156, samples from each sheep at 138 and 142 days were amplified in duplicate, one set with the GOBI and GOBF4 primer pair, and one set with the CC5 and CC6.2I primer pair, which are specific for the dehalogenase gene. Samples from each tube were run on 1.5% agarose, those from the CC primer reactions in duplicate because of concerns about carryover contamination from the first sample lane to the blank lane. The positive control template in each case was 1 µl of a 10^{-5} dilution of an OB156/pBHermF stationary phase culture.

Results.

a). Effects of Dietary Changes on Bacterial Populations.

Figures 3.2.4., 3.2.5., 3.2.6 and 3.2.7 show photographs of the gels of *B. fibrisolvens* AR10 and OB 156, and *P. ruminicola* AR20 and AR29 respectively. The scan results are tabulated in Table 3.2.10.

Densitometry measurements from photographs of PCR gels are linearly related to the logarithm of the number of bacteria in the PCR template. (see results of 3.2.1.c and the results of the scans of the positive controls of the PCRs of this experiment, Graph 3.2.1).

Notes on Results.

- It should be emphasised that because of differing efficiencies of PCR primer pairs, the amount of DNA/unit template produced in PCR reactions varied for each bacterial strain. Therefore the graphs indicate relative bacterial numbers within each strain, but do not offer comparisons between strains.

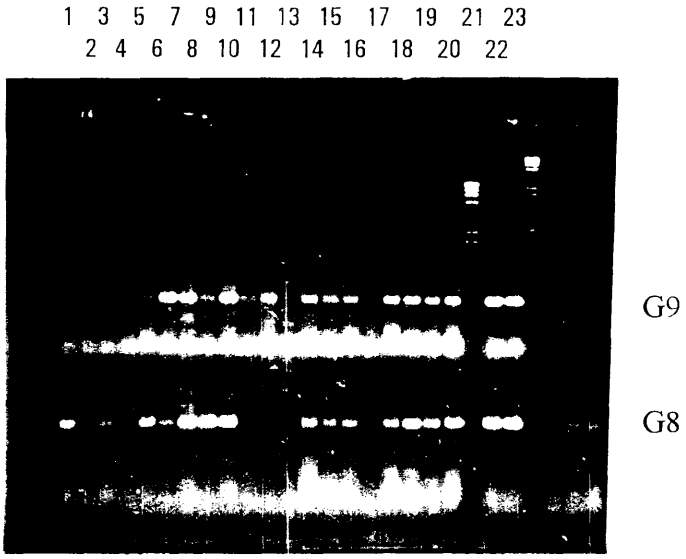
- The initial sample on day 1 was taken 2 hours after inoculation.

- It can be seen from the photographs, that the initial high level of inoculated bacteria dropped to low levels within the first two days, in many cases to undetectable levels. Also, in most cases, the numbers rose again to levels where they could be readily seen. The major exceptions were *B. fibrisolvens* OB156 in sheep G9, where numbers remained high, and P. ruminicola AR29 in sheep G8, where numbers remained below limits of detection for several weeks. All bacteria inoculated remained at intermittently detectable levels until the end of the experiment at 152 days.

- The four bacterial strains, all of which are xylanolytic, and could possibly be expected to compete for the same energy sources, differed markedly in their responses to alterations in diet.

Figure 3.2.4.

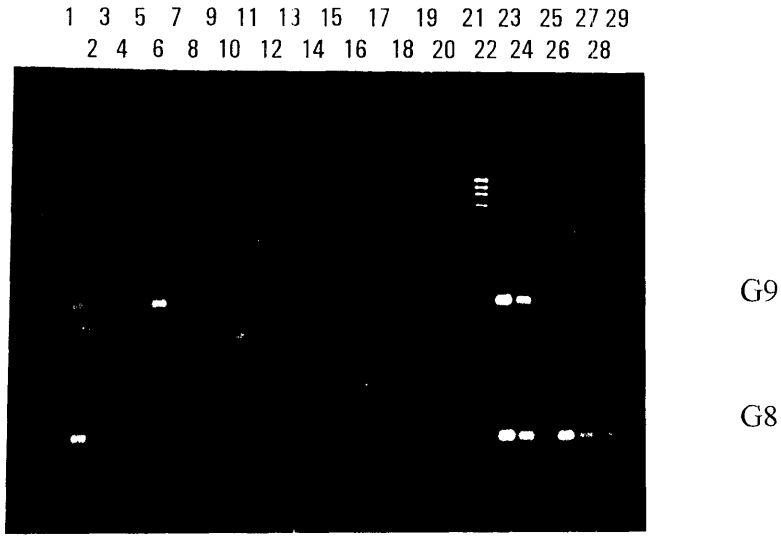
PCR products from Rumen Samples Us ng *B. fibrisolvens* AR10-Specific Primers.



Lanes 1-20 show rumen samples; lanes 21, G8, and 24, G9 show λ/*Hind* III size marker; lane 25 G9 shows negative control; lanes 22 and 23 for both G8 and G9 show 1 μl pDSS DNA at ~ 10^6 copies/ml.

Figure 3.2.5.

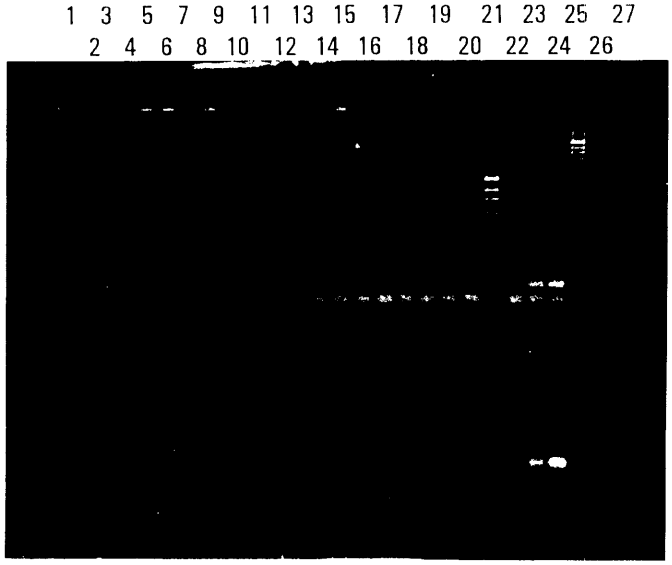
PCR products from Rumen Samples Using *P. ruminicola* AR20-Specific Primers.



Lanes 1-20 show rumen samples; lane 21 of G8 shows λ/*Hind* III size marker; lanes 22-23 of G9 show 1 μl pJW4 at 10^6 and 10^5 copies/ml; lanes 22-29 of G8 1 μl pJW4 at ~10^6, 10^5, 10^4 copies/ml and 1 μl *P. ruminicola* AR20 bacterial cells at ~10^6, 10^5, 10^4, 10^3 copies/ml

Figure 3.2.6.

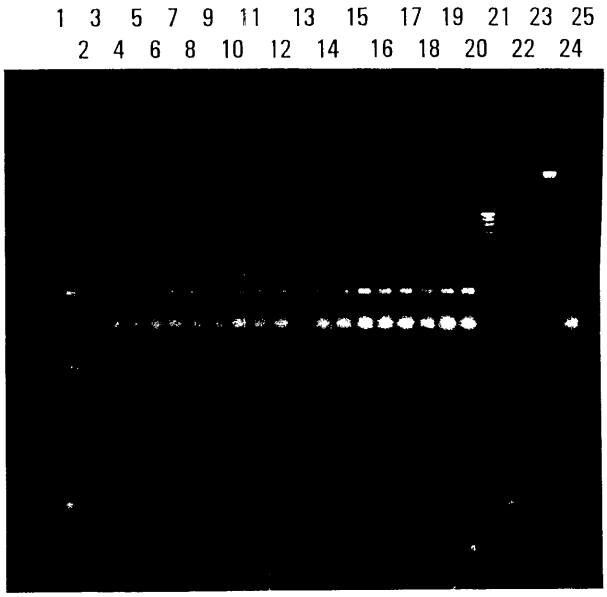
PCR products from Rumen Samples Using *P. ruminicola* AR29-Specific Primers.



Lanes 1-20 show rumen samples; lanes 21G8 and 25 G9 show *Hind* III/λ phage size marker; lanes 22-24 show 1 µl pGAR 11 DNA at ~ 10^5, 10^6 and 10^7 copies/ml; lanes 26 and 27 G8 show negative controls.

Figure 3.2.7.

PCR products from Rumen Samples Using *B. fibrisolvens* OB156-Specific Primers.



Lanes 1-20 show rumen samples; lane 21 G8. 24 G9 shows *Hind* III/λ phage size marker; lanes 21 of G8 and 23 of G9 show negative controls; lanes 22 G8 and 25 of G9 show 1 µl pGOB DNA at ~ 10^5 copies/ml.

Table 3.2.10.

Scan Readings of PCRs of Four Rumen Bacterial Strains.

Days Post Inoculation	Scan Readings Absorbtion Units mm							
	AR10 G8	AR10G9	AR20 G8	AR20 G9	AR29G8	AR29 G9	QB156 G8	QB156 G9
1	0.46	0.167*	1.008	0.707	0.507	0.57	1.074	0.698
2	0.1	0.094	0	0.122	0.188	0	0	0.332
4	0.194	0.085	0.166	0	0.141	0	0	0.504
10	0.07	0.12	0.095	0.15	0.033	0.181	0.398	0.464
17	0.43	0.223	0	1.005	0	0.14	0	0.475
24	0.177	0.67	0.274	0.07	0	0.139	0.215	0.511
31	0.71	0.65	0	0.417	0	0.181	0.158	0.501
38	0.667	0.243	0.477	0.219	0	0.092	0.165	0.298
59	0.57	0.747	0.232	0.275	0	0.187	0.414	0.935
63	0.107	0.129	0	0	0	0.25	0	0.546
67	0	0.557	0	0	0	0	0	0.603
70	0	0	0.23	0.539	0.156	0.086	0	0.325
74	0.27	0.365	0.155	0	0.319	0.225	0.195	0.567
77	0.17	0.284	0.335	0.179	0.333	0	0	0.626
80	0.212	0.305	0.207	0.079	0.374	0.239	0.275	0.833
84	0.08	0.119	0.452	0.07	0.314	0.32	0.155	0.832
138	0.388	0.488	0.089	0.276	0.348	0.268	0.425	0.623
142	0.545	0.426	0	0	0.327	0.176	0.594	0.784
145	0.357	0.412	0.080	0.233	0.312	0.253	0.305	0.738
149	0.472	0.537	0.073	0.193	0.295	0.189	0.281	0.92

* The day 1 result for AR10 G9 is anomalously low compared with other tests done on this specimen

B. fibrisolvens AR10 was present in at least one of the sheep, G9, before the start of the experiment , and probably at undetectable levels in the other, as the sheep had been penned together before the start of the experiment. While the sheep remained on a diet of oat chaff and lucerne, AR10 remained at low levels in G9, and moderate levels in G8. The removal of lucerne from the diet appeared to increase AR10 numbers in both sheep. A poor diet of wheat straw only, reduced AR10 numbers in both sheep, except for a rise on Day 67 in G9. The return to oat chaff and lucerne raised numbers again, but only to low or moderate levels. A diet of pure lucerne did not raise AR10 to the levels found when the sheep were on an oat chaff diet.

B. fibrisolvens OB156 colonised G9 at much higher numbers than G8, however the overall patterns for the two sheep were not dissimilar, though more definite in G8. Numbers were reduced on a wheat straw diet, and highest on a diet of pure lucerne.

P. ruminicola AR20 showed no clear pattern in response to changes of diet. The wheat straw diet appeared to reduce numbers over the first week, but in both sheep they rose again in the final wheat straw sample. There was no positive response to a lucerne diet. Numbers of AR20 seemed to fluctuate in both animals, but not noticeably in response to changes of diet, and there was not much similarity in the patterns shown by the two animals.

P. ruminicola AR29 numbers showed no marked response to feed in G9. However in G8, numbers declined to undetectable levels after the first 4 days, and remained below detectable levels until day 70, when the animal had been on a wheat straw diet for a week. Then they started to rise, and remained at detectable levels for the rest of the experiment. It was hypothesised that the wheat straw diet may have lowered the numbers of some competing organism or organisms, and enabled P. ruminicola AR29 to gain a competitive advantage which it maintained when the animal was returned to a better diet.

Note on Feeds Provided.

- Wheat straw and oat chaff contain about the same amount of hemicellulose material, about 30% of total dry weight, while lucerne contains much less, about 11%. (Feed Composition Tables, International Feedstuffs Institute, Utah,

USA, 1982). However lucerne has a high nitrogenous component, while wheat straw is very low in nitrogen.

b). Estimation of bacterial numbers.

Standards in the form of positive controls were available on the PCR photographs, they were scanned by laser densitometry and standard curves were plotted, as shown in graph 3.2.3.. These were then used to roughly estimate bacterial numbers.

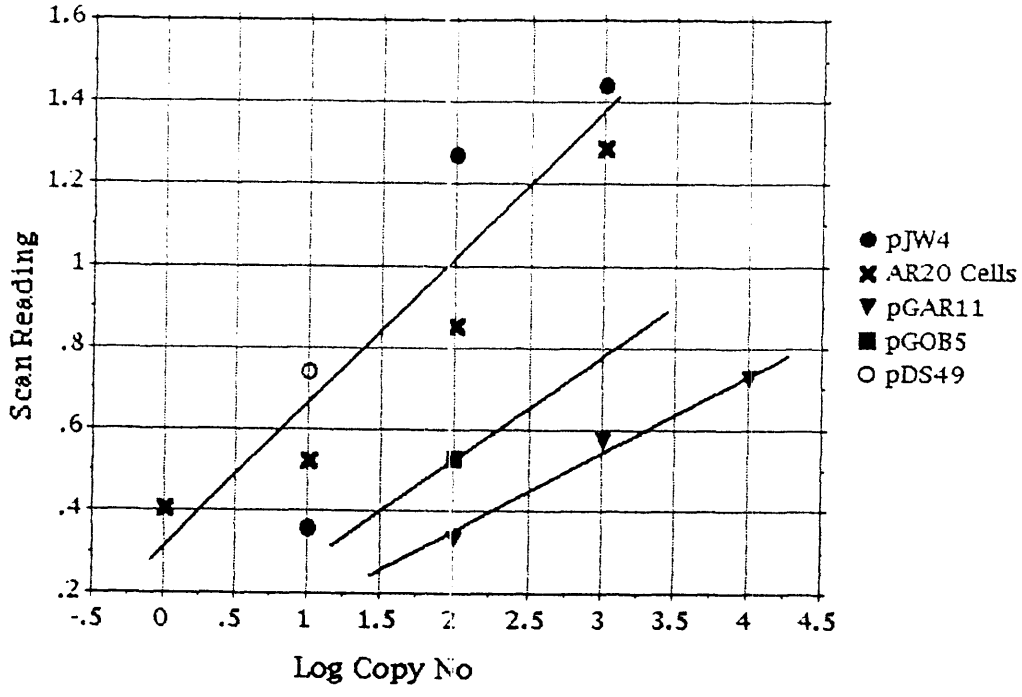
Table 3.2.11.

Positive Control Scan Readings.

Log Copy Nos	pJW4	AR20 cells	pGAR11	pGOB5	pDS49
0		0.404			
1	0.357	0.521			0.747
2	1.27	0.854	0.337	0.532	
3	1.44	1.29	0.577		
4			0.727		

Graph 3.2.3.

Plot of Log Copy No per Reaction vs Scan Reading.



Notes on Graph 3.2.3.

(i). Since both plasmid and bacterial cells were used as standards, figures are given as copy number of template DNA rather than bacterial cell. In all cases there is one copy of the target sequence on the bacterial genome. Usually, there will be a single copy of the genome in each bacterial cell.

(ii). The line through the single point for pGOB5 scan is highly speculative. The position of the single point suggests a sensitivity between that of the *P. ruminicola* AR20 detection primers, and the *P. ruminicola* AR29 detection primers.

(iii). The position of the pDS63 point was calculated from a mean of four scan results for the single copy number figure. The position of the point suggests that the pDS63 primer pair has similar sensitivity to the *P. ruminicola* AR20 primer pair.

(iv). The lowest possible level of detection for bacteria was 1 cell/µl, (i.e. 10^3/ml), as the sample size for PCR is 1 µl of rumen contents.

(v). The lowest reliable densitometer reading was approximately 0.1 Au.mm, which, from Graph 3.2.6., corresponds to a lower limit of detection of:

~ 1 copy no/µl for *B. fibrisolvens*_AR10 and *P. ruminicola* AR20;

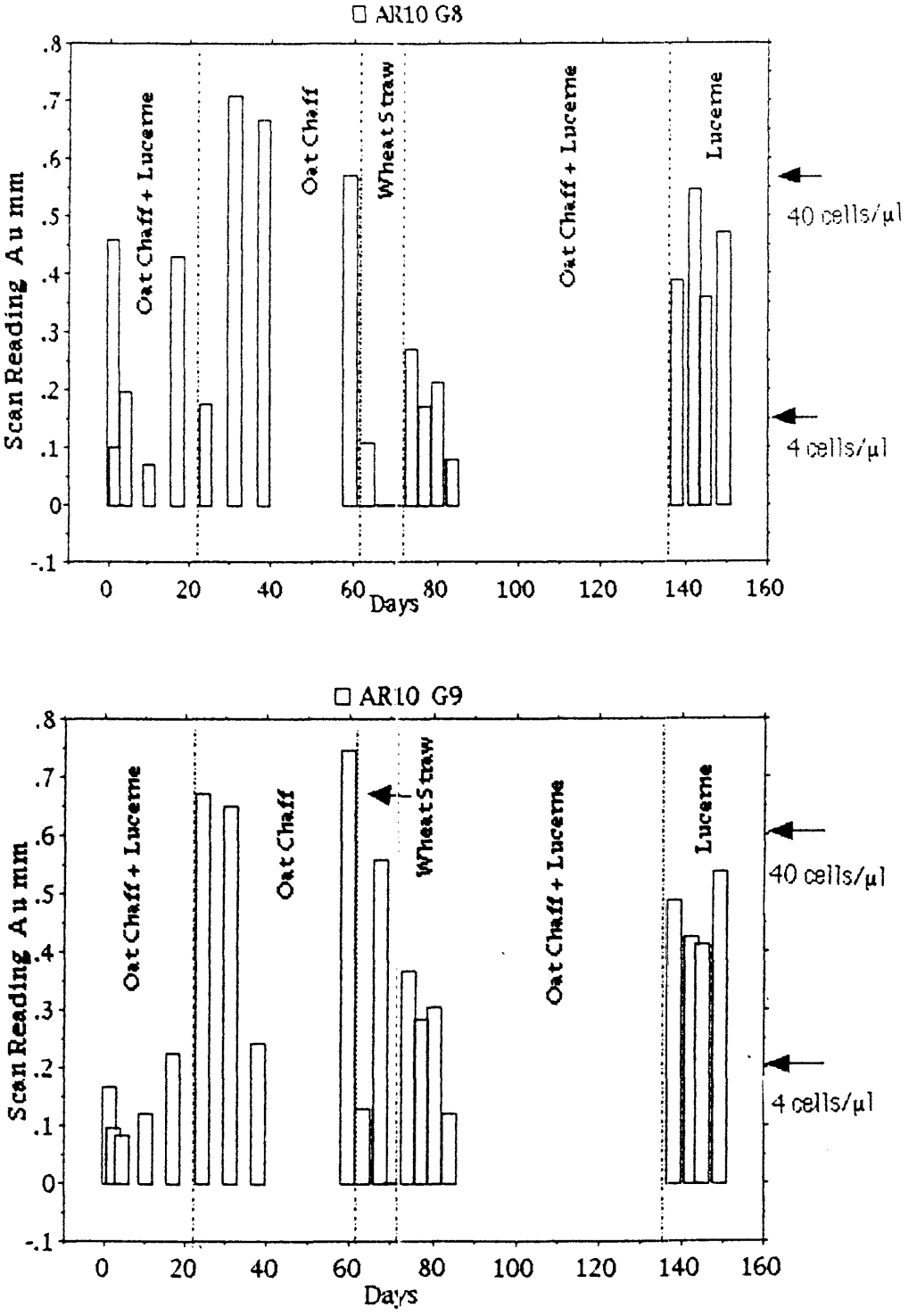
~ 2 copy no/µl for *B. fibrisolvens* OB156;

~ 6 copy no/µl for *P. ruminicola* AR29.

(vi). Adjustment was needed to relate the standards, diluted in water, to the rumen samples. From results plotted on Graph 3.2.1, a correction factor of four was required to compensate for the inhibiting effect of rumen material on PCR amplification.

Graph 3.2.4.

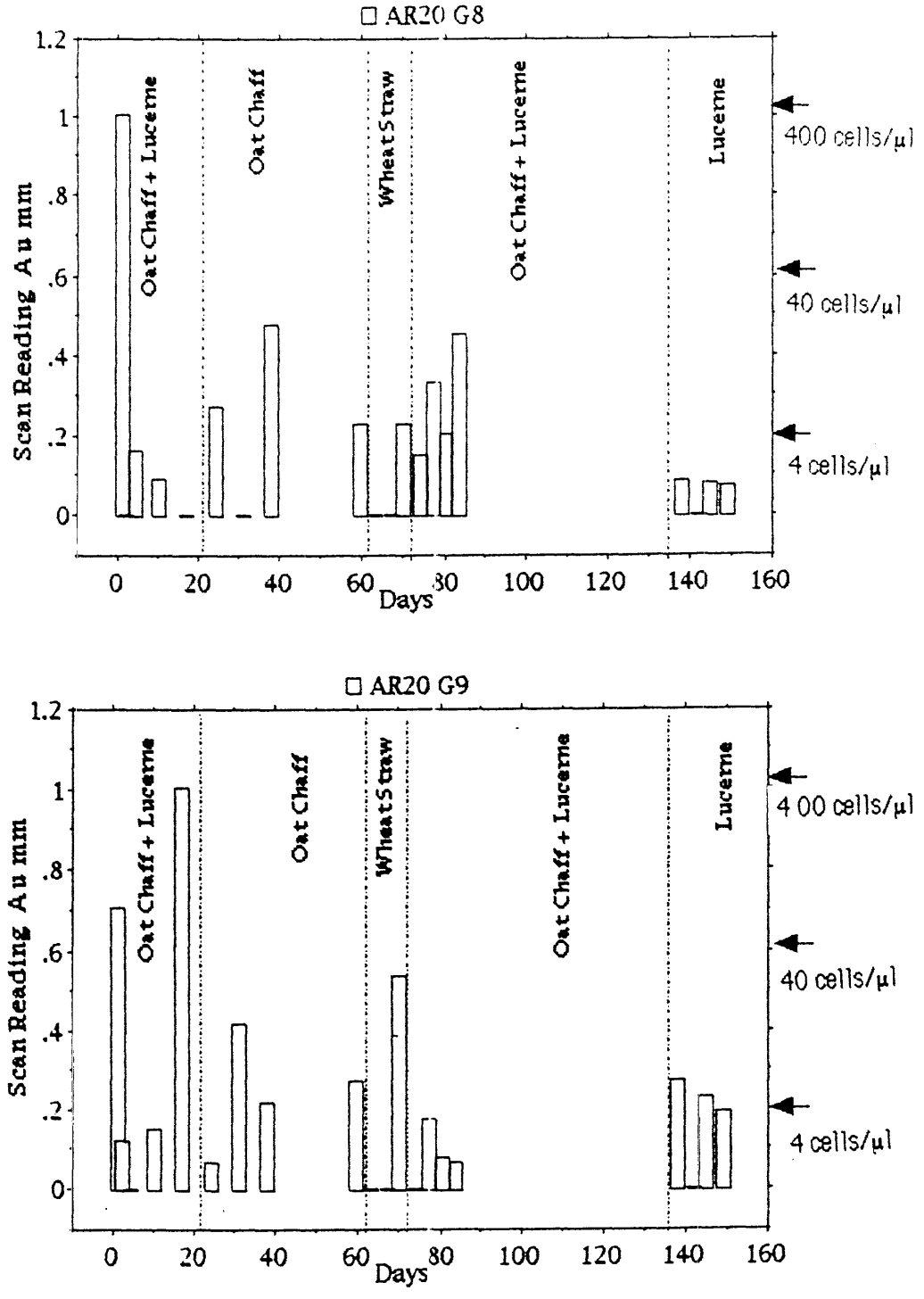
Bacterial Cell Numbersin Sheep Rumen Samples – *B. fibrisolvens* AR10.



Cell numbers are estimates from Graph 3.2.3. Copy numbers of rumen bacterial cells have been corrected for the effects of rumen sample dilution by multiplying the results obtained from the standard plot by 4.

Graph 3.2.5.

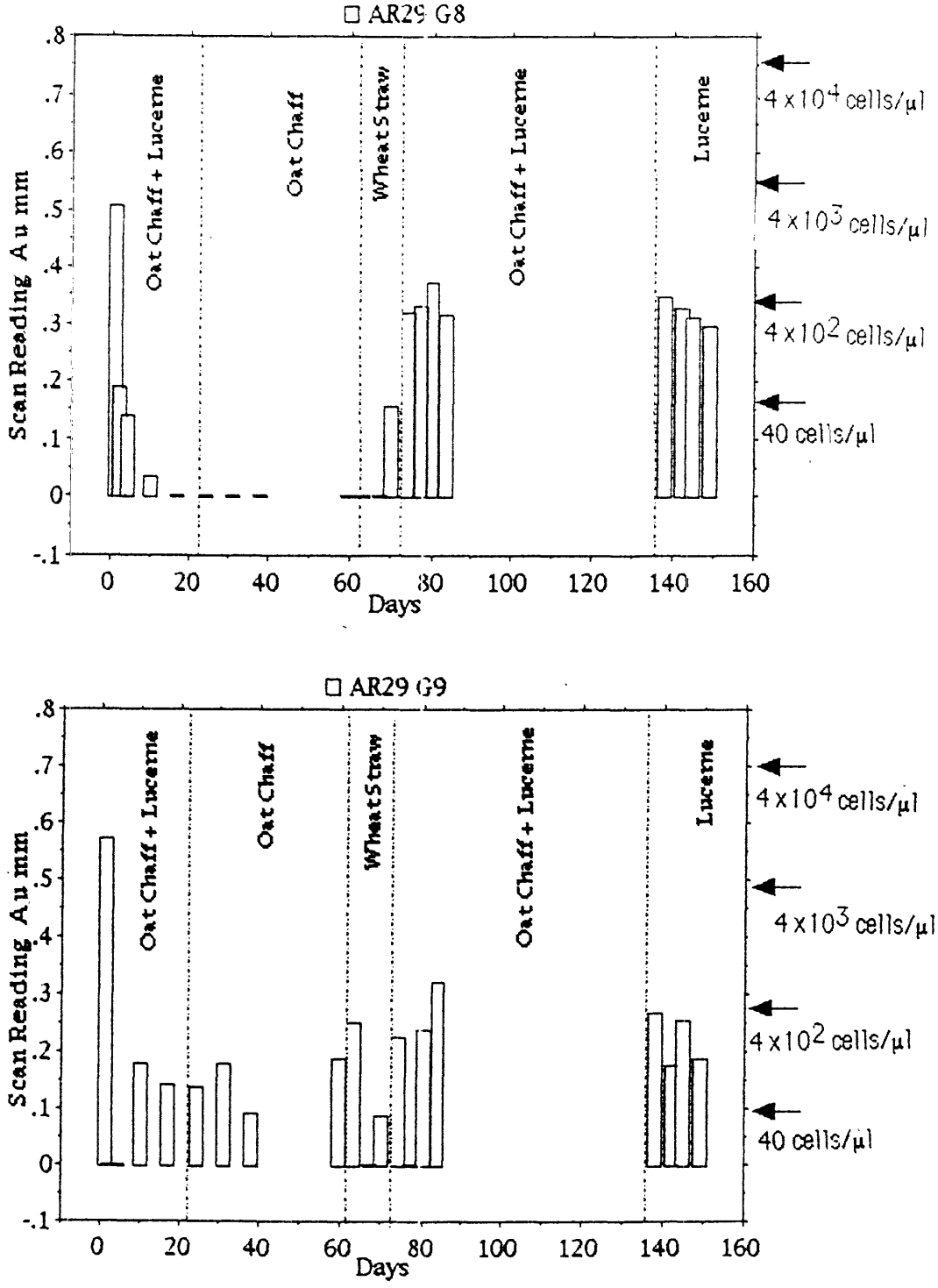
Bacterial Cell Numbersin Sheep Rumen Samples – *P. ruminicola* AR20.



Cell numbers are estimates from Graph 3.2.3. Copy numbers of rumen bacterial cells have been corrected for the effects of rumen sample dilution by multiplying the results obtained from the standard plot by 4.

Graph 3.2.6.

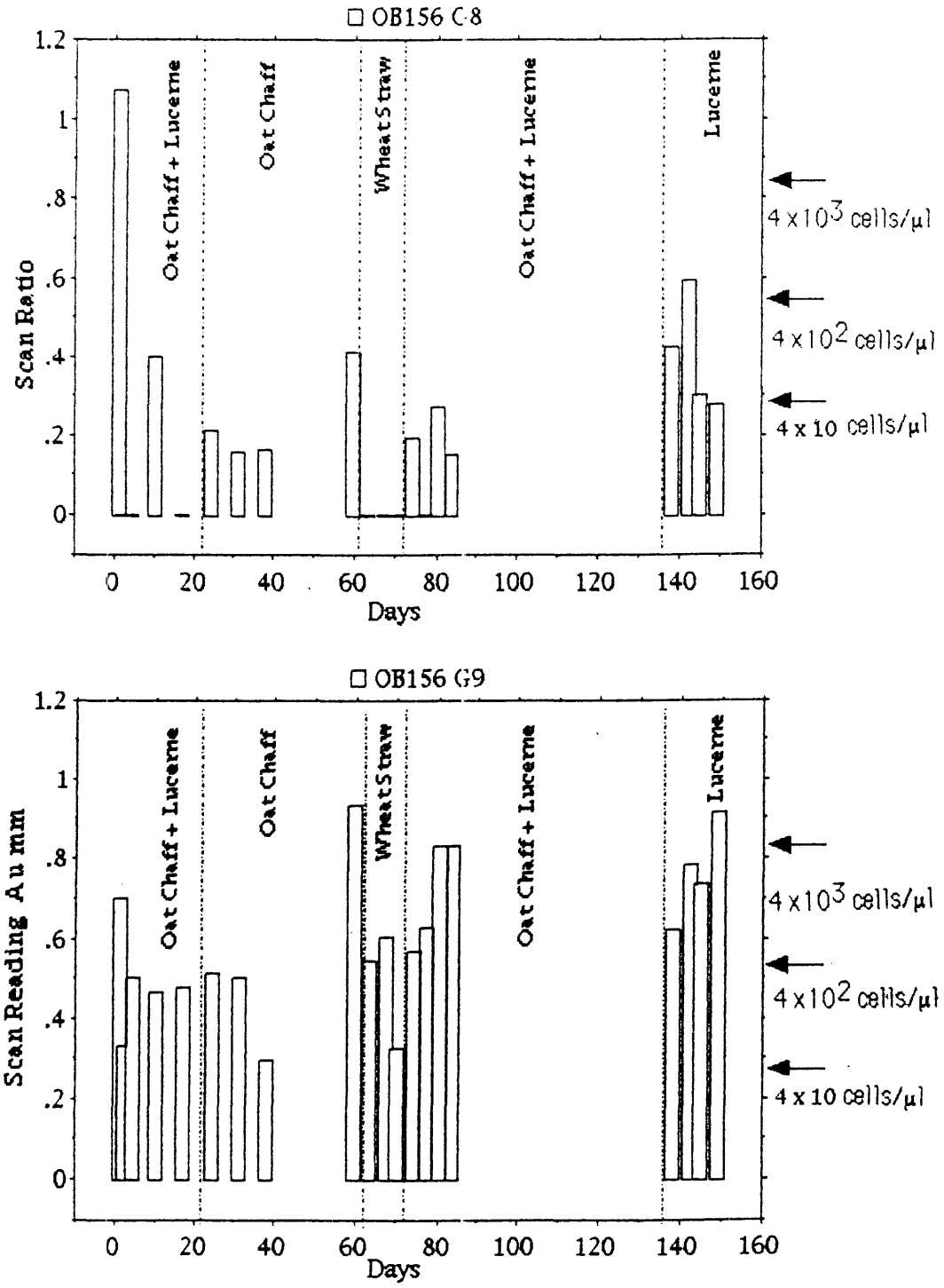
Bacterial Cell Numbersin Sheep Rumen Samples – *P. ruminicola* AR 29.



Cell numbers are estimates from Graph 3.2.3. Copy numbers of rumen bacterial cells have been corrected for the effects of rumen sample dilution by multiplying the results obtained from the standard plot by 4.

Graph 3.2.7.

Bacterial Cell Numbersin Sheep Rumen Samples – *B. fibrisolvens* OB156.



Cell numbers are estimates from Graph 3.2.3. Copy numbers of rumen bacterial cells have been corrected for the effects of rumen sample dilution by multiplying the results obtained from the standard plot by 4.

Using the standard curves of Graph 3.2.3., the scan readings of Table 3.2.10 show the following population variations in the four organisms tracked:

B. fibrisolvens AR10 numbers varied from ~ 4 x 10^2/µl, to less than 1/µl in G8 and G9.
B. fibrisolvens OB156 numbers varied from ~ 4 x 10^2/µl to less than 2/µl in G8 and ~4 x 10^3/µl to ~ 50/µl in G9.
P. ruminicola AR20 numbers varied from ~ 4 x 10^2/µl to less than 1/µl in G8 and G9, however the top figure came from an anomalously high result on day 17. More normal values for both sheep were up to ~ 30/µl.
P. ruminicola AR29 numbers varied from ~ 4 x 10^2/µl to less than 2/µl in G8 and G9

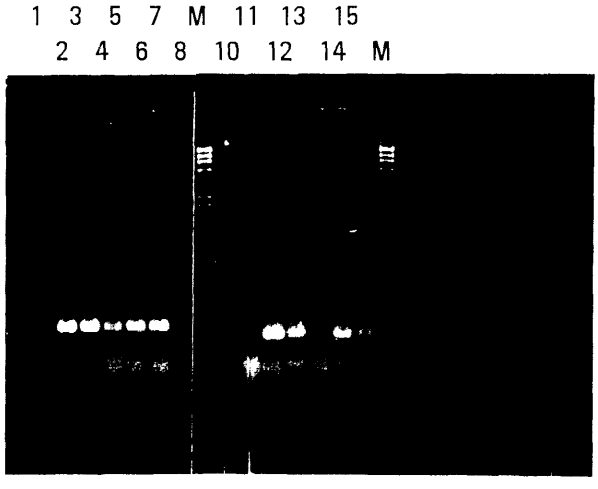
Despite the reservations detailed above, it seems reasonable to suggest that the populations of *P. ruminicola* AR29 and *B. fibrisolvens* OB156 stabilised at levels higher than those of *B. fibrisolvens*. AR10 and *P. ruminicola* AR20. Population estimates for *B. fibrisolvens* AR10 and *P. ruminicola* AR20 were of the order of 10^4/ ml, and those of *P. ruminicola* AR29 of the order of 10^5/ml, and *B. fibrisolvens* OB156 over 10^6/ml in G9.

Stability of pBHermF in *B. fibrisolvens* OB156.

Fig 3.2.8 shows the results of the PCR of samples from days, 138 and 142, from G8 and G9, using GOBF4 and GOBI primers for *B. fibrisolvens* OB156 and CC5 and CC6.2I primers for the dehalogenase gene. It can be seen that the dehalogenase gene was still present after 20 weeks, and that the amount of DNA produced from the dehalogenase gene varies in the same way as the amount of DNA produced from OB156, suggesting that the dehalogenase gene and OB156 co-varied, and therefore the majority of OB156 cells still carried the pBHermF plasmid, and that the gene had not spread to other rumen bacteria

Figure 3.2.8.

Rumen Samples Probed with Dehalogenase and OB156 Specific Primers



Lane 1 shows negative control for CC primers; lane 2 shows positive control for CC primers; lanes 3 and 4 show rumen samples from G8 or days 138 and142 amplified with CC primers ; lanes 5 and 6 show rumen samples from G8 or days 138 and142 amplified with CC primers. Lane 10 shows negative control for GOB primers; lane 11 shows positive control for GOB primers; lanes 12 and13 show rumen samples from G8 on days 138 and142 amplified with GOB primers lanes 143 and15 show rumen samples from G8 on days 138 and142 amplified with GOB primers. Lanes 8 and 16 show λ/*Hind* III size marker.

3.3. DISCUSSION.

The sequences chosen for PCR amplification proved to be specific for the original target bacteria, or those of closely related genotypes. The specificity results supported the results of the DNA hybridisation work of Hudman and Gregg (1989). They also correlated with results obtained by comparing genomic DNA restriction patterns (Gregg *et al,* 1987. It seems that strains of bacteria, which are detected by the same PCR probes, are very closely related genetically. The success of the *B. fibrisolvens* AR10, *P. ruminicola* AR29 and *B. fibrisolvens* OB156 probes showed that 21mer to 24mer primers, complementary to random fragments of genomic DNA, can be used as specific probes for the target bacteria. This required PCR to be performed under conditions of fairly high stringency. (An annealing temperatures of 65°C or higher proved to be stringent enough to prevent amplification when the primer and template were mismatched by a single nucleotide. See Chapter 6.).

The *B. fibrisolvens* AR10 DS49 probe system was less sensitive than the *P. ruminicola* AR20 system. This may be because the sequence chosen for amplification from AR10 contained a 12 bp stem loop with a free energy of dissociation of -16.6 Kcal/mole at sequence positions 95 -107 (see appendix). Since only a short piece of AR10 genomic sequence, 362 bases, was cloned into the pDS49 plasmid,, it was not possible to avoid this secondary structure. The AR20 sequence was free of secondary structure.

The hypothesis that internal secondary structure had an adverse effect on the sensitivity of PCR probes was supported by the fact that primer pairs complementary to the *B. fibrisolvens* AR10 genomic DNA fragment in the plasmid pDS63, which was free of secondary structure, appeared to have sensitivity equal to that of the pJW4 *P. ruminicola* AR20 probe. The *B. fibrisolvens* OB156 genomic sequence in pGOB5 contained two internal stem loops of ΔG -15.2 and -17.6, and the primer pair complementary to this was of lower efficiency than the pJW4 and pDS63 systems. The *P. ruminicola* AR29 genomic DNA in pGAR11 had an internal stem loop with ΔG of -18.6, and it appeared to be of lower efficiency than any of the others (see Table 3.2.11). These figures show an inverse relationship between the free energy of association of internal stem loops, and the sensitivity of the primer pairs, in terms of amplification efficiency.

Time did not permit the sequencing of other plasmids containing genomic fragments of *P. ruminicola* AR29 and *B. fibrisolvens* OB156 so as to try to obtain fragments free of secondary structure in the hope of developing more sensitive probes.

However, even the pGAR system was able to detect template copy numbers down to approximately 2/20 µl PCR reaction, equivalent to 2 x 10^3/ml of test sample.

It should also be emphasised that sensitivities, as measured by densitometry scans of Polaroid negatives of ethidium bromide stained electrophoresis gels of the reaction products, do vary between different PCR amplifications using the same sets of primers. These variations arise from such factors as slight variations in reaction mixtures between the different PCR amplifications, age of *Taq* enzyme, differences between batches of film, and in the ethidium bromide used to stain the electrophoresis gels. This necessitates the use of standard templates with each set of amplifications.

The logarithmic relationship between template DNA copy number and scan results is thought to be due to the response of the Polaroid film, rather than the ethidium bromide fluorescence. Digital scanning of the fluorescent gel would possibly give a linear relationship

The limit of detection of 10^3 cells/ml, when using whole cells as template, was dictated by the amount of rumen sample that could be used as template in a 20 µl PCR reaction without losing sensitivity. The sensitivity could be increased a little by using larger PCR reaction volumes, however this is expensive, and PCR volumes greater than 50 µl are not commonly used, at least in part because, as reaction volume rises, there are increasing problems in achieving rapid, uniform heat changes throughout the amplification sample. It is also possible that greater sensitivity could be obtained using extracted DNA rather than whole cells. However this would be at the cost of increasing the risk of contamination and the time taken to prepare samples.

For the detection of numbers of bacteria lower than 10^3/ml, rumen contents could be cultured in enrichment media. While selective media for specific rumen bacterial strains do not exist, media with xylan as the only carbon source would favour *Prevotella* and *Butyrivibrio* species, and experimentation with inoculation media showed that lack of haemin in the medium prevented growth of *P. ruminicola* AR20, but not *P. ruminicola* AR29. It is probable that with some experimentation, enrichment media with greater selectivity could be devised. Another strategy would be to sample plant fragments only

for fibrolytic bacteria, as it has been shown [Section 3.2.2(ii)] that *B. fibrisolvens* is predominantly found in the plant fraction of rumen contents.

The use of whole cells rather than extracted DNA for PCR amplification represented a marked saving in time and effort, and also helped to keep down the possibility of cross contamination, as there was much less manipulation of the samples. It seems probable that the use of whole cells would give a more representative picture of bacterial populations than would extracted genomic DNA, as the extraction process is possibly biased towards recovery of DNA from more readily lysed bacterial species, as well as including unknown amounts of DNA from plant fragments, protozoa and fungi.

The rumen material sampled, fluid plus small suspended plant fragments, provided a reasonably representative sample of rumen contents that could be easily collected, and used as PCR template with minimal preparation. The fact that at least one of the target bacterial strains adhered to plant material necessitated the inclusion of plant material in the samples to be tested. The idea of sampling larger fragments of plant material, which could then be macerated in some way prior to testing was rejected on the grounds of complexity of sample preparation, problems with sample reproducibility and difficulty in preventing sample contamination. To determine total rumen populations of organisms, it would be necessary to ensure sampling of all rumen environments, including bacteria adhering to the rumen epithelium, which was not feasible.

The three bacterial strains investigated in the North Queensland rumen survey were all obtained from sheep rumen at the University of New England in Armidale. This experiment showed that bacteria of similar genotype to two of the strains, *P. ruminicola* AR20 and *R. albus* AR67, were also present in both cattle and sheep in North-West Queensland. AR20 is a strain currently being used for attempted genetic modification. It is therefore of considerable interest that similar genotypes have widespread distribution, as it suggests that genetically modified versions of AR20 have a good chance of being retained in the rumen of both sheep and cattle over a wide range of climate and conditions.

The results of the Headingley *B. fibrisolvens* experiment showed:

- That *B. fibrisolvens* AR10 could colonise cattle rumen.
- That the organism was able to spread through the herd.
- That the level of AR10 found in any one animal fluctuated markedly over time.

- That after an initial rise in the numbers of animals showing the presence of AR10, there was a steady decline until the 268 day sample. However, numbers had risen again at the final sampling date, when 37% of animals tested carried the organism. This was well down on the results for the first two months, but showed a significant increase over the results at 268 days.

The results correlated well with rainfall and the type of feed available. Headingley has a monsoonal climate with a summer "Wet", and little rain for the rest of the year. It seems probable that the introduction of *B. fibrisolvens* AR10 in the wet season allowed it to establish itself under conditions which favoured the nutritional requirements of this organism. Under dry conditions, the level of AR10 in the herd fell, to rise again at the next wet season (the final sample was taken just after the first heavy rain of the Wet), when green feed was once more available (Graph 3.2.2.), suggesting that the organism remained in the rumen of at least some members of the herd, even under unfavourable conditions.

This suggested that for AR10 to be a suitable carrier for introduction of genetic modifications in an area where green feed is not readily available, it may require modifications that give it a competitive advantage, such as the introduction of cellulase genes.

The fact that levels of *B. fibrisolvens* AR10 fluctuated within and between animals (Table 3.2.9.) demonstrated that a single sampling of any one animal in a herd is not enough to establish the absence of a specific bacterial strain. Also, the results from the inoculation of multiple bacterial strains, particularly from the introduction of P. ruminicola AR29 into G8, show that it is possible for a particular bacterial strain to exist at very low levels in the rumen, and rise again to a significant proportion of the rumen population when conditions become favorable. This agrees with the reports of Jones *et al.* (1985) on detoxification of *Leucena* by rumen bacteria.

There was a possibility that the observed population fluctuations of AR10 in the Headingley cattle were not a true reflection of bacterial abundance, but were due to sampling error. However if this suggestion had been true, the bacterial numbers in the multiple tracking experiment would have been expected to co-vary within any one sample. No such co-variance was seen.

The experiments on the introduction of multiple strains of rumen bacteria into sheep confirmed the conclusions of the Headingley experiment, that foreign rumen bacteria may be readily and lastingly introduced into the rumens of animals. Evidence was also produced that a bacterium transformed with a plasmid does not appear to be at a competitive disadvantage in the rumen environment, even though the plasmid is not known to carry any gene that provides an advantage to the host organism under rumen conditions. This particular plasmid, pBHermF, also remained stably in its host bacterium over a period of 5 months. There did not appear to be any evidence of loss of plasmid from *B. fibrisolvens* OB156.

Changes in the populations of the various bacterial strains in response to changes in diet were demonstrated. However it was obvious that dietary factors were not the only ones that influenced bacterial populations. What those other factors may be can only be guessed, but predation by protozoa, and lysis by bacteriophage, are possible influences, which may work directly, or work indirectly by removing competitors.

In summary, PCR was shown to be a highly successful technique for tracking rumen bacteria. The results from the four separate strains introduced into sheep rumen show clearly that each strain of a particular bacterial "species" has distinct features and capabilities that respond uniquely to differing conditions within the rumen ecosystem. There is obvious potential to vastly extend the present knowledge of ruminal ecology, by determining which particular bacterial strains flourish under which dietary conditions. This may provide the potential to manipulate rumen bacterial populations to enable ruminants to adapt quickly and without stress to different feed and forage sources.