

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**PCR probes for ammonia hyper- producing bacteria
in the rumen of New Zealand ruminants**

A thesis presented in partial fulfilment of the
requirements for the degree of

Master of Science
in
Nutritional Science

at Massey University, Albany Campus,
New Zealand.

Shanmuganathan Manickavasagar

2000

Abstract

Competitive PCR (cPCR) primers were developed to detect and enumerate 5 hyper ammonia-producing (HAP) bacteria previously isolated from New Zealand ruminants, and 3 previously described HAP bacteria, *Clostridium aminophilum*, *C. sticklandii* and *Peptostreptococcus anaerobius*. Primers were designed by aligning 16S ribosomal RNA gene sequences and identifying unique site for each bacterium. Primers were matched as closely as possible in terms of length, G+C content and T_m to either the universal eubacterial forward (fd1*) or reverse (rd1*) primers to anchor the PCR at either the 5' or 3' end of the 16S rRNA gene. Primer specificity was tested in amplification reactions with DNA extracted from 35 bacterial isolates, mostly from the rumen. The primers designed for isolates C2 and D5 produced amplified PCR products only with their respective target DNAs. Primers developed for isolates S1, D4 and *P. anaerobius* also amplified DNA from closely related species, *P. asaccharolyticus*, *Fusobacterium necrophorum* and isolate D1, respectively, in addition to their respective target DNAs. Internal controls were developed for each of the chosen primers by creating deletions in the amplified target DNA using restriction endonuclease digestions and religating the terminal fragments. The deleted internal control fragments were reamplified and cloned into the PCR cloning vector pGEM-T. Cloned internal control DNAs were coamplified with known amounts of their respective target DNAs to generate standard curves so that unknown samples could be quantitated. DNAs extracted from rumen samples from sheep fed a diet of chaffed lucerne and infused with either monensin or buffer were probed for HAP bacteria using the cPCR probes. The results showed that isolates C2, D5, S1 and *C. sticklandii* and *C. aminophilum* were below the detectable limits of the cPCR technique and their population could not be enumerated. The absence of any PCR amplifiable DNA of these organisms in the rumen

samples was confirmed by conventional PCR in the absence of internal control DNAs, by additional purification of rumen DNAs followed by reamplification, and by preamplifying rumen DNA with the universal eubacterial primers *fdl** and *rdl** prior to PCR with primers specific to each organism. However the D4/*F. necrophorum* and D1/*P. anaerobius* probes showed detectable populations in the samples. *In vivo* the D1/*P.anaerobius* population in the rumen ranged from 3 to 7×10^8 cells ml^{-1} . Monensin showed no inhibitory effect on the D1/*P.anaerobius* population, which maintained steady levels throughout the sampling period. D4/*F.necrophorum* populations ranged from 3×10^8 to 1.4×10^9 bacteria ml^{-1} . Monensin had little effect over the first 48hr compared to control sheep but after 72hr D4/*F.necrophorum* populations increased and finally reached 1.4×10^9 bacteria ml^{-1} at 96 hrs.

ACKNOWLEDGEMENTS

I express my most sincere appreciation to my Chief supervisor Professor T. N. Barry, Institute of Veterinary, Animal and Biomedical Sciences, Massey University, for allocating necessary funds, and granting me the opportunity to complete my post graduate research studies at Massey University, Palmerston North.

I gratefully acknowledge my supervisor, Dr. Graeme Attwood, Rumen Microbiology Unit, AgResearch, Grasslands, Palmerston North, for his patience, constructive criticisms, discussions and advice of immense value in all aspects of the project, including the preparation of this thesis.

The advice and support given to me by the Rumen Microbiology Unit technical staff, Graham Naylor, Kerry Reilly, and Diana Burgess, is greatly appreciated.

My special thanks to Dr. Keith Joblin, Dr. Lucy Skillman, Dr. Abdul Molan, and Dr. B.R.Min for their friendship, assistance and advice in the scanning and quantitation of rumen DNA.

My sincere thanks to AgResearch, Grasslands, Palmerston North for allowing me to carry out research studies in the Rumen Microbiology Unit.

I am greatly indebted to my wife Nalini and my daughters, Baheerathi, and Arunthathi, for their patience, trust and support towards achieving my goal. This thesis is dedicated to my late father and my mother, who had been of great inspiration to my life and shall remain in my fondest memories for years to come.

CONTENTS

Abstract	i
Acknowledgements	iii
Contents	iv
List of Figures	vii
List of Tables	viii
Chapter 1. Introduction	1
Chapter 2. Literature Review	4
2.1. Introduction	4
2.2. Nitrogen metabolism in the rumen	4
2.2.1 Peptide metabolism in the rumen	5
2.2.2 Amino acid metabolism	6
2.3. Effect of monensin on protein degradation, peptide accumulation and deamination by mixed ruminal microorganisms	9
2.4. Enrichment and isolation of ruminal bacteria with high specific activity of ammonia production	12
2.5. Phylogeny of the ammonia hyper-producing ruminal bacteria	18
2.6. Enumeration of ammonia-hyper producing bacteria in the rumen	18
2.7. Screening of New Zealand ruminants for HAP bacteria	21
Chapter 3. Materials and methods	24
3.1. Bacterial strains	24
3.2. Chemicals	25
3.3. Media	25
3.3.1. HAP(Hyper ammonia-producing) Medium	25
3.3.2. Salt solution 1(for HAP medium)	26
3.3.3. Salt solution 2(for HAP medium)	26
3.3.4. Vitamin solution (for HAP medium)	26
3.3.5. Microminerals (for HAP medium)	27
3.3.6. Complete Carbohydrate (CC) medium	28
3.3.7. Mineral solution 1 (for CC medium)	28

3.3.8. Mineral solution 2 (for CC medium)	29
3.3.9. Volatile fatty acid solution (for CC medium)	29
3.3.10 Cysteine Sulphide solution (for CC medium)	29
3.3.11 Lab M fastidious anaerobe agar for <i>E. tardum</i>	29
3.3.12 Brain Heart infusion (BHI) medium for <i>E. tardum</i>	30
3.3.13 PYG (Peptone, yeast extract and glucose) medium for <i>Eubacterium tardum</i>	30
3.3.14 Salt solution (for PYG medium)	30
3.3.15 SOB medium	31
3.3.16 SOC medium	31
3.3.17 LB broth	31
3.4 Buffers and solutions	32
3.4.1 Gel-loading Buffer	32
3.4.2 TAE Buffer	32
3.4.3 TE Buffer	32
3.4.4 GTE Buffer	32
3.4.5 Saline-EDTA	32
3.4.6 Mineral salts Buffer	33
3.5. Bacterial culturing	33
3.6. Rumen sampling	33
3.7. Phenol/chloroform/isoamyl alcohol extractions	33
3.8. Ethanol precipitation	34
3.9. DNA extraction	34
3.9.1 Enzymatic lysis	34
3.9.2 Physical disruption	34
3.10. DNA quantitation	35
3.11. Agarose gel electrophoresis	35
3.12. Extraction of DNA from agarose	35
3.13. Restriction endonuclease digestions	36
3.14. Ligations	36
3.15. Cloning PCR products	36
3.16. Transformations	36
3.17. Plasmid DNA minipreparations	37

	3.18. Preparation of HAP isolates for sensitivity testing	38
	3.19. Quantitation of PCR products	38
	3.20. Animal Trial	38
Chapter 4.	Results	40
	4.1. Introduction	40
	4.2. Results	41
	4.2.1 16S sequence analysis and primer design	41
	4.2.2 Primer optimisation	48
	4.2.3 Primer specificity	49
	4.2.4 Construction of internal controls	51
	4.2.5 Amplification of rumen DNA with specific primers	52
	4.2.6 Standard curve construction for strain D4 and <i>P. anaerobius</i>	54
	4.2.7 Analysis of rumen samples	57
Chapter 5.	Discussion and Conclusions	61
	5.1 Discussion	61
	5.2 Conclusions	72
Chapter 6.	References Cited	74

List of figures

Figure	4.1.	Alignments of C2 16S rDNA	42
Figure	4.2.	Alignment of D1 16S rDNA sequence	43
Figure	4.3.	Alignment of D4 16S rDNA sequence	44
Figure	4.4.	Alignment of D5 16S rDNA sequence	45
Figure	4.5.	Alignment of S1 16S rDNA sequence	46
Figure	4.6.	C2 internal control construction	52
Figure	4.7.	D4 standard curve	56
Figure	4.8.	<i>P. anaerobius</i> standard curve	57
Figure	4.9.	Coamplification of <i>P. anaerobius</i> with DNA extracted from rumen samples	59
Figure	4.10.	Strain D4/ <i>F. necrophorum</i> and D1/ <i>P. anaerobius</i> populations in the rumen infused with monensin or with buffer	60

List of tables

Table	3.1.	Bacterial strains	24
Table	4.1.	Primers	48
Table	4.2.	Optimized PCR conditions for primer amplification	49
Table	4.3.	Primer specificity	50
Table	4.4	Construction of internal controls	53

Chapter 1. Introduction

Ammonia Hyperproducing (HAP) bacteria are non-proteolytic, peptide and amino acid fermenting rumen microorganisms, producing large amounts of ammonia during growth. These bacteria are however unable to use carbohydrates as energy source. Earlier work in the rumen assumed that deamination was carried out by a large population of the principal species of ruminal bacteria which were shown to produce ammonia from protein and protein breakdown products. However Chen and Russell (1988 and 1989a), and Russell et al., (1988 and 1991) have shown that these bacteria did not have a specific activity of ammonia production high enough to account for the high rate of ammonia production by the mixed bacterial populations in the rumen. They isolated a group of bacteria that were present in very small populations, but had a specific activity of ammonia production that was very much higher than that of the commonly isolated rumen bacteria. These HAP bacteria were also very sensitive to monensin, and as ammonia production was greatly reduced in ruminants fed with monensin, it was deduced that these bacteria must be responsible for significant ammonia production in the rumen. *Peptostreptococcus anaerobius*, *Clostridium aminophilum*, and *Clostridium sticklandii* were the species isolated and these were unable to ferment any carbohydrates but used amino acids as their main source of energy, nitrogen and carbon sources. These HAP rumen bacteria have been extensively studied in the Northern Hemisphere where animals are fed mainly dried forage and concentrates. There is very little information on the prevalence of HAP bacteria or their contribution to ammonia production in ruminants grazing fresh forage under New Zealand conditions. Forages grown in New Zealand are rich in protein but are low in soluble carbohydrates. New Zealand ruminants are very vulnerable to nitrogen

loss as ammonia and urea. Preliminary studies by Attwood et al. (1998) found that between 5.2 % and 11.6 % of bacteria in rumen samples were able to grow in a medium containing amino acids and peptides as the sole nitrogen and carbon source. This medium was not completely selective for obligate peptide and amino acid utilising bacteria as it also allowed the growth of some carbohydrate-utilising bacteria. However the study did isolate organisms that had properties of HAP bacteria and further characterisation showed that most of them were distinct from the monensin-sensitive ammonia producing bacteria isolated from ruminants in the Northern Hemisphere. The lack of a suitable selective medium for studying these organisms therefore created a need for an alternative method to identify and quantitate these organisms from rumen samples.

Analysis of bacterial nucleic acids is a comprehensive and reliable method for bacterial identification. The 16S ribosomal RNA gene provides a compact source of genetic information, which enables rapid determination of bacterial phylogenetic relationships. The 16S ribosomal RNA gene also contains sites with unique sequences, which can be utilised for designing probes. 16S rRNA probes have been used previously to follow *in vivo* and *in vitro* populations of rumen bacteria by probing RNA extracted from samples in a slot-blot format (Stahl et al., 1988; Briesacher et al., 1992; May et al., 1993; McSweeney et al., 1993; Odenyo et al., 1994a; Odenyo et al., 1994 b). However, this method is not very sensitive and small population of bacteria may be missed. Also quantitation of bacteria using this method is expressed only as a proportion relative to the total RNA present as determined by universal rRNA probes and therefore gives no direct indication of numbers of bacteria present. The sensitivity limitation of 16S ribosomal RNA probes may be overcome by adapting this method for use with the Polymerase

Chain Reaction (PCR). Using 16S rDNA sequences to design specific primers and using these primers in PCRs can retain specificity of the 16S ribosomal RNA probes while dramatically increasing sensitivity. As PCR has the potential to amplify one molecule of DNA 10^6 times (Saiki et al., 1998), a large increase in probe sensitivity is possible. As PCR has been previously used to follow populations of bacteria in the environment in a quantitative manner using competitive PCR (cPCR, Bej *et al.*, 1991, Bej and Mahubani, 1992, Reilly and Attwood, 1998), it could be used to study HAP bacterial populations in rumen samples.

Therefore the aim of this study is to use 16S ribosomal RNA sequences in designing specific oligonucleotides which will be tested as specific primers to enumerate HAP rumen bacteria using a cPCR technique. Competitive PCR primers will be designed for the five HAP bacteria isolated from New Zealand ruminants (Attwood et al., 1998) to enable the populations of these organisms to be estimated *in vivo*. Probes will also be designed for *C. aminophilum*, *C. sticklandii* and *P. anaerobius*, isolated from ruminants in the Northern Hemisphere, to estimate their prevalence in New Zealand ruminants. Because the previously described HAP bacteria are known to be monensin-sensitive, the cPCR probes developed in this study will be applied to the quantitation of HAP bacteria in rumen samples from animals receiving an intraruminal infusion of monensin in a controlled indoor animal trial.

Chapter 2. Literature Review

2.1 Introduction

New Zealand ruminants graze pasture which tends to be high in protein, but low in energy-producing carbohydrates throughout the year (Johns, 1955). Most of the available protein in the pasture is degraded (up to 80%) to peptides, amino acids and ammonia. As the rate of ammonia production usually exceeds its rate of utilisation in microbial protein synthesis, it accumulates within the rumen. Ammonia can be absorbed through the rumen wall into the portal circulation and carried to the liver where it is converted into urea. Some urea is recycled via the saliva back into the rumen but most is excreted in the urine. Excessive ammonia formation in the rumen is therefore a wasteful process ending in inefficient utilisation of forage nitrogen by the animal (Nolan, 1975).

2.2 Nitrogen metabolism in the rumen

The main source of ruminal nitrogen is the feed protein but it can also enter the rumen in the form of nitrates, nitrites, nucleic acids, urea and mucosal proteins. Most of the feed protein is degraded in the rumen but some may bypass the rumen into the abomasum for further digestion. Bacteria, protozoa and fungi all degrade nitrogen sources in the feed but among these, bacteria are the most active proteolytic organisms in the rumen (Brock et al., 1982; Nugent and Mangan, 1981). Many species of bacteria have been identified as proteolytic (Abou Akkada, 1963; Blackburn et al., 1962; Fulghum and Moore, 1963; Wallace and Brammall, 1985). These proteolytic bacteria are also capable of degrading peptides and amino acids, and this had led to the general assumption that these bacteria are also responsible for the degradation of dietary proteins to ammonia.

Feed protein is broken down mainly by proteinases and peptidases which are cell-associated (Blackburn and Hobson, 1960; Kopecny and Wallace, 1982; Prins et al., 1979). The predominant proteinase activity within a ruminant fed dried forage and concentrate is a cysteine-type protease (Wallace and Brammall, 1985) which is often associated with the rumen bacterium *Prevotella ruminicola*. Recently it has been proposed that this species be

reclassified into *P. ruminicola*, *P. bryantii*, *P. brevis* and *P. albensis* (Avgustin et al., 1997). Their proteolytic activity is similar to that of the mixed bacterial fraction acting on dried forage and concentrate diets. Isolates of *Ruminobacter amylophilus*, *Butyrivibrio fibrisolvens* and *Fusobacterium* species have also been found have high proteolytic activity, but which is of the serine proteinase type (Wallace and Brammall, 1985), which is not the prevalent proteolytic activity of the mixed bacterial population. *Streptococcus bovis* is another important proteolytic bacterium with a low proteolytic activity, but with a very high leucine aminopeptidase activity (Wallace and Brammall, 1985).

2.2.1 Peptide metabolism in the rumen

Mixed ruminal bacterial populations break down different peptides at different rates. The main feature of amino acid sequences that disposes some peptides to be more resistant than others to degradation appears to be gly-gly, pro-x or x-pro residues at the N-terminus or greater than one acidic residue in the peptide (Wallace et al., 1990a; Yang and Russell, 1993; Wallace, 1996). Peptides present in ruminal fluid several hours after feeding may have survived degradation, as they are naturally N-formylated or-acetylated. The mixed ruminal bacteria showing predominantly amino peptidase activity cleave dipeptides rather than single amino acids from peptide chains (Wallace et al., 1990a, 1993). These enzymes are called dipeptidyl peptidases (Webb, 1992). The peptide break down in rumen fluid is a 2-step process with dipeptidyl peptidase releasing dipeptides from oligopeptides, followed by separate dipeptidases which cleave the dipeptides to amino acids. *P. ruminicola* is the only ruminal bacterium that possesses high dipeptidyl peptidase activity (Wallace and McKain, 1991; McKain et al., 1992; Wallace et al., 1997). *P. ruminicola* like the mixed rumen population has low aminopeptidase activity against amino acyl-*p*-nitroanilide substrates but high activity against dipeptidyl-*p*-nitroanilide substrates. It also cleaves dipeptides from longer peptides as the first step in peptide hydrolysis (Wallace et al., 1993c).

Diet seems to influence the type of peptide break down. If *S. bovis* prevails then its leucine aminopeptidase activity will predominate and amino acids may be cleaved singly from

peptide chain rather than in pairs (Wallace and Brammall, 1985). Many species of bacteria and protozoa have dipeptidase activity. *P. ruminicola* shows activity against a wide range of dipeptides. Similarly *Megasphaera elsdenii* has high activity against dipeptides (Wallace and McKain, 1991).

The products of protein breakdown in the rumen are oligopeptides, peptides and amino acids. In the presence of sufficient energy, amino acids will be incorporated into bacterial proteins and peptide break down would not be an inefficient process. However when peptide break down exceeds the rate at which it could be assimilated or if energy is not sufficient, then peptide catabolism contributes to excessive ammonia production and poor nitrogen retention. Most studies of ^{14}C -amino acids and peptides have indicated that the mixed microbial population preferentially incorporates peptides than free amino acids (Wright, 1967; Prins et al., 1979; Cooper and Ling, 1985). Pure culture studies with *P. ruminicola* showed that larger peptides were preferred for growth (Pittman and Bryant, 1964), and that peptides up to a molecular weight of 2000 were taken up much more rapidly than free amino acids (Pittman et al., 1967). However Armstead and Ling (1993) found that amino acids were preferred over peptides in rumen fluids from some sheep than in others. They suggested that there may be a dependence on diet. Amino acid transport is more predominant than peptide transport in *S. bovis*. Ling and Armstead (1995) found that free amino acids were the preferred form of amino acids incorporated by *S. bovis*, *Selenomonas ruminantium*, *Fibrobacter succinogenes* and *Anaerovibrio lipolytica* while *P. ruminicola* preferred peptides. Therefore the observed preference for peptides by the mixed bacterial population in most studies reflects the large numbers of *P. ruminicola* which could comprise more than 60% of the total bacterial population in sheep receiving grass silage (Van Gylswyk, 1990).

2.2.2 Amino acid metabolism

There is very little free amino acid in rumen fluid and most of it is found intra-cellularly (Lewis, 1955, Wright and Hungate, 1967; Wallace, 1979) but the breakdown products of amino acids, including volatile fatty acids (VFA) are found in high concentration in rumen fluid (El-Shazly, 1952a). El-Shazly (1952b) showed that the source of ruminal ammonia is

largely amino acids but it could also be produced from urea and nitrates. The extent of amino acid accumulation varies with the diet. Amino acids accumulate from rapidly degraded protein (Broderick and Wallace, 1988). Lysine, phenylalanine, leucine, isoleucine, arginine and threonine are broken down faster, whereas valine and methionine are more stable. The carbon skeletons arising from deamination give rise to a variety of volatile fatty acids. These provide energy for the microorganisms. There can be Stickland-like reactions whereby pairs of amino acids are metabolised and provide energy via coupled oxidation and reduction reactions (Barker, 1981).

Most of the early work on ruminal amino acid metabolism was carried out with mixed suspensions of rumen bacteria or rumen fluid. Few data were available on the specific organisms involved. The few studies conducted identified bacteria such as *Selenomonas ruminantium* (Bryant, 1956) and *Bacteroides ruminicola* (Bryant et al., 1958b). These bacteria were shown to degrade cysteine and casein hydrolysates. Lewis and Elsdén (1955) showed that *Peptostreptococcus elsdenii* fermented L-serine, L-threonine and L-cysteine to produce ammonia, volatile fatty acids, hydrogen and carbon dioxide. Bladen *et al.*, (1961) undertook a comparative study of pure cultures and freshly isolated rumen bacteria to identify the predominant organisms degrading protein hydrolysates and producing ammonia. They concluded that, on the basis of the number of bacterial strains and the amount of ammonia produced, *Bacteroides ruminicola* was of primary importance in ammonia production. They also found that *Selenomonas ruminantium* was a significant ammonia producer and *Peptostreptococcus elsdenii* may be of importance under certain conditions of high grain feeding. Some strains of the genus *Butyrivibrio* may also be involved in ammonia production. They found that many ammonia-producing species of bacteria were among the predominant bacteria of young calves. Of a total of 271 bacterial strains isolated and presumptively identified, 75 (28%) produced 1 μ M or more ammonia from the Trypticase medium. The most numerous group (37 strains) was presumptively identified as *Bacteroides ruminicola*. This group also contained the organism, which was responsible for the production of most amounts of ammonia. Bladen et al., (1961) also had indicated the possibility that other bacteria, not among the predominant bacteria would be of importance in ammonia production and their presence could be demonstrated by using culture media

containing protein hydrolysates as the main source of energy for growth. In these batch culture studies, ammonia production was measured after 96 hours. However the magnitude of increase was not substantial and less than 25% of the casein hydrolysate was converted into ammonia. Russell (1983) also has shown that the ammonia generation rate was slow and within the range reported by Bladen et al., (1961). Hungate (1966) reported that ammonia production in the rumen could be as high as 375mg per litre and this rate is 250 times the level determined by Russell (1983). Russell attributed this to the total cell density in the culture, as laboratory cultures usually contain 10^8 bacteria per ml whereas it could be as high as 10^{10} bacteria per ml *in vivo*. Hungate (1966) also reported that he had encountered rumen bacteria able to digest casein that did not require carbohydrates for growth. However he failed to isolate any of these bacteria.

Russell (1983) conducted a series of experiments with *Bacteroides ruminicola* and incubated these organisms in continuous cultures so that their growth rate, maintenance energy and growth yields could be measured under steady state conditions. His primary objective was to find out whether the rate of amino acid deamination was influenced by growth rate of the bacteria and to estimate the energy production from amino acid fermentation. His study showed that the growth rate of *Bacteroides ruminicola* was significantly improved by Trypticase, acetate and C4 – C5 fatty acids. Inclusion of 15g per litre of Trypticase resulted in maximum ammonia production. *B. ruminicola* was unable to grow on peptides alone as it was unable to utilise peptides at a rate fast enough to meet its maintenance requirement. During growth phase there was little change in the concentration of extra cellular ammonia but thereafter it increased at a linear rate. In a continuous culture, Trypticase fermentation to ammonia was inversely proportional to the dilution rate but even at a dilution rate of 0.087 per hour, less than 5 % of the available Trypticase was degraded. Despite energy being potentially available from deamination of amino acids by mixed rumen bacteria (Russell et al., 1983) neither mixed rumen bacteria nor pure cultures of *P. ruminicola* or *M. elsdenii* gave much higher yields^{of ammonia} as a result of amino acid fermentation (Russell and Baldwin, 1979; Wallace 1986a). It was suggested from pure culture work that the energy produced would contribute only to the maintenance energy requirement of the bacteria. However there was clear evidence accumulating that a

relatively small population of atypical bacteria may be responsible for a significant amount of amino acid deamination that occurs in a mixed rumen population. Russell and his colleagues at Cornell University showed that the predominant rumen bacteria, which had been identified as ammonia producers by Bladen et al (1961), did not have sufficient activity to account for the observed rates of ammonia production by mixed population in their cattle. They isolated from these animals, a group of bacteria, which possessed a specific activity of ammonia production that was an order of magnitude greater than that of the other species (Chen and Russell, 1988, 1989a; Russell et al., 1988, 1991). These bacteria namely *Clostridium sticklandii*, *Clostridium aminophilum* and *Peptostreptococcus anaerobius* were asaccharolytic relying on fermentation of amino acids for growth. Moreover they were highly sensitive to monensin. Since ammonia concentration was lower when this ionophore was fed in the diet, it was deduced that they must be significant ammonia producers *in vivo*.

2.3 Effect of monensin on protein degradation, peptide accumulation and deamination by mixed ruminal microorganisms.

Monensin, when included as a feed additive in the cattle feed, depresses ammonia production in the rumen, accompanied by an increased flow of peptides from the rumen into the abomasum. Thus monensin played a role in “protein sparing” effect on the cattle feed in the rumen. Early work by Bladen et al., 1961, suggested that Gram negative bacteria were most responsible for ammonia production. It was concluded that *B. ruminicola* was the most ammonia-producing bacterium in the rumen. However *B. ruminicola*, like many other Gram negative bacteria, is resistant to monensin (Chen and Wolin, 1979), and this species cannot account for the rapid rates of ammonia production (Hino and Russell, 1985). Recently isolated Gram-positive ruminal bacteria that had much higher specific activities of ammonia production (Russell et al., 1988; Chen and Russell, 1989a) were sensitive to monensin. Therefore this inhibitory effect of monensin on bacteria showing high specific activity of ammonia production was thought to explain its “protein sparing effect” on the animal feed in the rumen (Dinius et al., 1976; Van Nevel and Demeyer., 1977; Russell and Martin, 1984).

Monensin is effective primarily against Gram-positive bacteria (Russell and Strobel, 1989). The protonophore, tetrachlorosalicylanid completely inhibited the growth of *B. ruminicola* B₁₄ but it did not cause a greater decrease in ammonia than monensin. Thus the “protein sparing” effect of monensin is thought to be due to its effect on Gram positive bacteria. Newbold et al., 1990 reported that *B. ruminicola* cultures that were adapted to the ionophore tetronasin took up oligopeptides at a slower rate than the unadapted cells. The unadapted cells took up peptide nitrogen 10-fold more slowly than the ammonia nitrogen production of mixed cultures (Hino and Russell, 1985). This adaptation, seemed to involve alteration in outer membrane porins, and affected only the uptake of larger peptides (four amino acids or greater).

Monensin is a metal-proton antiporter which catalyses a rapid outflow of potassium out the ruminal bacteria and inflow of sodium and protons into the bacteria (Russell, 1987). Since the new ammonia hyper producing isolates use sodium gradients as a driving force for transport (Chen and Russell, 1989b, 1990), ammonia production is inhibited by monensin. Most bacteria maintain an electrical potential across the cell membrane, and this potential can contribute to the driving force if the overall transport process is electrogenic. The electric potential is rapidly dissipated by the protonophore, tetrachlorosalicylanide (TCS). Two of the recent isolates from the rumen could use the electric potential as a driving force (Chen and Russell, 1990). Inability of monensin-TCS treatment to completely inhibit ammonia production indicates that the bacteria are using some other method of transport. In some cases ATP hydrolysis can serve as a driving force, but recent work indicates that facilitated diffusion may also be involved in amino acid transport (Chen and Russell, 1989b, 1990). Since large amounts of non-amino nitrogen, and non-protein nitrogen accumulated during *in vitro* incubations, facilitated diffusion cannot be excluded (Chen and Russell, 1990).

The influence of ionophores on peptide metabolism by ruminal microorganisms is not yet clear. Prolonged exposure to ionophores in continuous cultures leads to accumulation of peptides (Whetstone et al., 1981). It is also the case in sheep rumen *in vivo* (Wallace et al., 1993a). Ionophores also inhibited incorporation of small quantities of labelled peptides by

mixed microorganisms *in vitro* (Armstead and Ling, 1993). However acute addition of monensin or tetranasin to rumen fluid *in vitro* had no effect on the peptide breakdown (Wallace et al., 1990b). This suggests that adaptation was required for the inhibitory effect on peptide breakdown. It is possible that rumen microorganisms alter their response to ionophores causing a change in peptide metabolism. *Prevotella ruminicola* decreases its membrane permeability when exposed for long periods to ionophores. This slows its rate of peptide breakdown (Newbold et al., 1992). This may in turn alter the peptidolytic activity of the whole rumen population.

Yang and Russell (1993) extensively studied the effect of monensin on the specific activity of ammonia production by ruminal bacteria and disappearance of amino nitrogen from the rumen. When mixed ruminal bacteria from cows were incubated *in vitro* with Trypticase (0.5g of N per litre) on different days, ammonia was produced at a linear rate of 27.4 ± 2.2 nmol/mg of protein per min for 6 hours. The most probable number (MPN) of bacteria in ruminal fluid which could utilise mixed carbohydrates as an energy source was 6.5×10^8 per ml, but only 4.1×10^6 /ml could use Casamino acids as energy for growth. When the cows were fed 350mg of monensin per day, the specific activity of ammonia production decreased to 17.2 ± 1.5 nmol/mg of protein per min. Monensin had little effect on the MPN of carbohydrate-utilising bacteria (7.0×10^8 /ml), but the MPN of the bacteria utilising Casamino acids decreased 10-fold to 4.2×10^5 /ml. Since the MPN of carbohydrate-utilising bacteria did not decline, it appeared that the amino acid-sparing effect of monensin was due to a decreased number of the Gram-positive, monensin-sensitive bacteria which could utilise amino acids and peptides as an energy source for growth. These bacteria were recently identified as *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, and *Clostridium aminophilum* (Paster et al., 1993).

In vitro experiments showed that ruminal bacteria utilized proline- containing peptides slowly (Yang and Russell, 1993) and the proline content of the protein hydrolysate was inversely correlated with the ruminal disappearance rate. Monensin decreased the ruminal disappearance rate of all protein hydrolysates irrespective of their proline content. Since less proline-containing peptides are utilised one would expect an increase in the outflow of

proline from the rumen. No such trend was observed. As the amino acid composition of residual amino acid nitrogen was similar to that of the original material, proline seemed to protect a variety of other amino acids from ruminal degradation.

2.4 Enrichment and isolation of ruminal bacteria with high specific activity of ammonia production.

Russell et al., (1988) used the same strains of *Bacteroides ruminicola* and *Megasphaera elsdenii* used by Bladen et al., (1961) and the results from their preliminary experiments showed that the specific activity of ammonia production was less than 20nmol of ammonia mg^{-1} protein min^{-1} . However mixed rumen bacteria, which were obtained directly from the rumen, had specific activity greater than 25nmol mg^{-1} protein min^{-1} . Russell et al., (1988) proceeded further to enrich the media and isolate rumen bacteria with high specific activities of ammonia production. When mixed ruminal bacteria were inoculated into media containing lactate, dulcitol, pectin, or xylose and transferred in a semicontinuous fashion, the specific activity of ammonia production was higher than in the mixed bacterial control. Over the next 14 days the ruminal bacterial control showed a specific activity ranging from 25 to 92nmol of ammonia per mg of cell protein per min, but the enrichments showed specific activities as much as 12- fold higher values. Lactate enrichment culture showed an immediate and maximum increase in specific activity and large cocci predominated in this medium. Further enrichment of the above culture media containing the same energy source failed to show any further increase in the specific activity of ammonia production but was in fact lower than that of the ruminal fluid control. This indicated that those bacteria with the capacity for high ammonia production were not isolated. When 15g of Trypticase per liter was used as the sole enrichment and nitrogen source, there was a very rapid increase in the specific activity of ammonia production. Even though carbohydrates as additional source of energy were not included, the organisms in the medium were able to grow and persist in the semicontinuous culture. This enrichment culture contained primarily cocci and motile rods.

A coccus (strain C) approximately 1 μm in diameter was isolated. It was unable to utilise any of the carbohydrates tested as a sole energy source and grew extremely slowly when

these carbohydrates were added to cultures containing low concentrations of Trypticase. It grew rapidly under anaerobic conditions with Trypticase (15g/liter) as the only energy source and produced, after 24 hours of incubation, 29.0mM ammonia, 5.4-mM acetate, 2.2 mM *iso*-butyrate, 3.9 mM *iso*-valerate-2-methylbutyrate, 2.3mM valerate and 8.9 mM *iso*-caproate. The coccus produced large amounts of hydrogen sulphide in the presence of Trypticase or cysteine, but was unable to use pyruvate, lactate, or mannitol and could not reduce hydrogen sulphide. It did not produce ammonia from urea and could not grow under aerobic conditions without a reducing agent. It grew at 25°C or 39°C but not at 45 °C. There was no spore formation, the cells were non-motile, stained Gram positive and were unable to grow in the presence of 5ug/ml monensin. The G+C content of the DNA was 36.8 mol%.

The rod (strain R) was obtained from a rough irregular colony. Optical densities increased if glucose, fructose, maltose, cellobiose, trehalose, sorbitol or salicin was added to the medium, which contained low concentrations of Trypticase. However it did not grow when ammonia was substituted for Trypticase. Incubations with Trypticase (15g/litre) yielded 35.9mM ammonia, 5.4mM acetate, 1.3mM *iso*-butyrate, 6.7 mM isovalerate, 2-methylbutyrate and 5.0mM methanol after 24 hours. It was unable to produce ammonia from glutamate or glycine. The rod was able to liquify gelatin, digest casein and produce hydrogen sulphide from amino acids. Sulphate reduction and urease, catalase or lipase activity could not be demonstrated. There was no growth under aerobic conditions. Growth was seen at 25, 39 and 45 °C. The cells were motile and initially contained sub-terminal spores. With repeated transfers most of the spores disappeared, but growth still occurred after the culture was subjected to 80 °C for 10 minutes. It was also Gram positive and sensitive to monensin. The specific activities of ammonia production of strains C and R were 346 and 427nmol mg⁻¹ protein min⁻¹, respectively.

Since most other ruminal bacteria are unable to use Trypticase as a sole energy source for growth (Russell, 1983), the ability of the ammonia-producing bacteria to grow on Trypticase enabled their enumeration. When the ruminal contents were serially diluted on 3 different days, the most probable number of bacteria growing on Trypticase was

0.9×10^8 . Tubes containing a mixture of glucose, maltose and cellobiose (1g of each per liter) as well as Trypticase (15g per liter) had the most probable number of 8.0×10^8 . High dilution tubes containing only Trypticase and showing significant growth had an abundance of cocci, but motile rods were also seen. If the tubes were incubated at 45°C , a temperature tolerated by the rods, no growth was seen at dilutions higher than 10^3 suggesting that the rods are not present in high numbers in the rumen.

On the basis of shape, Gram stain, capacity to produce endospores, and survival at 80°C , inability to grow under anaerobic conditions, absence of catalase activity, inability to reduce sulphate to sulphide, the rod (strain R) fits the taxonomy of the genus *Clostridium*. Based on the presumptive identification listed in Bergy's Manual of Systematic Bacteriology this strain most closely resembled *Clostridium sporogenes*. However unlike *C. sporogenes*, the strain R did not produce butyrate and did not show any lipase activity. *C. sporogenes* did not produce acid from cellobiose, but strain R showed significant increase in optical density when cellobiose was added to the cultures. Based on the absence of spores, Gram staining, inability to use a variety of carbohydrates as energy sources, failure to produce lactate, inability to produce catalase activity, lack of growth under aerobic conditions, the coccus (strain C) fitted the descriptions of the genera *Peptococcus* or *Peptostreptococcus*. The G+C content analysis, a characteristic differentiating the two genera, indicated that this strain should be classified as a *Peptostreptococcus*. In many ways the strain C resembled *Peptostreptococcus anaerobius* but it had a G+C content of 36.8% which was higher than the value reported for *P. anaerobius*. In addition, *P. anaerobius* weakly fermented glucose and maltose. Strain C also showed some similarities to *Peptostreptococcus heliotrinreducens*, but here too it had many differences like its inability to grow at 45°C , and the ability to produce branched-chain fatty acids.

The newly named *Peptostreptococcus* was able to deaminate only a few amino acids whereas mixed ruminal bacteria deaminated most amino acids. Therefore it was suggested that there were other bacteria in the rumen which had high specific activities of ammonia production. Further work by Chen and Russell (1988) led to the isolation of two more ammonia-producing, monensin-sensitive ruminal bacteria, which grew, rapidly with amino

acids as the sole energy source. When mixed ruminal bacteria were inoculated into medium containing 15g Trypticase per liter of the medium as the sole energy source, growth was observed at dilutions as high as 10^8 . These enrichments contained cells showing 5 distinct morphologies: crescent shaped cells, long slim rods, short rods, irregularly shaped rods, and cocci. The crescent-shaped organisms and long slim rods produced less than 3mM ammonia after 24 hours of incubation, but the short rods (strain SR) and the irregularly shaped cells (strain F) produced more than 29mM ammonia. The cocci also produced large amounts of ammonia and were similar to the previously described ruminal *Peptostreptococcus*.

The small short rods (strain SR) were isolated from smooth white circular, but slightly irregular colonies. These grew faster and to a higher optical density with Trypticase than with Casamino acids. The products of Trypticase fermentation were 42.2mM ammonia, 22.0mM acetate, 3.3mM propionate, 2.8mM *iso*-butyrate, 8.8mM butyrate and 5.3 mM *iso*-valerate. When carbohydrates were added to Trypticase-limited (2g/liter) cultures there was hardly any increase in optical density. SR did not hydrolyse gelatin or digest casein, produced hydrogen sulphide, but did not show any catalase, urease, lipase or lecithinase activity. It grew at 25 and 39°C but not at 45 °C. It grew under anaerobic conditions. The cells were motile, Gram variable, and sensitive to the ionophore monensin (5uM). No spores were observed and it had a G+C content of 37.3%. Arginine, serine, lysine, and glutamine (15g/liter) were rapidly deaminated to ammonia at rates of 425, 351, 271, and 158 nmol/ mg of protein per min. respectively. When strain SR was inoculated into media containing either arginine, serine, lysine, glutamine or theonine (15g/liter) as the sole source of energy, growth was observed after one transfer indicating that single amino acids could be used as the sole source of energy. If a small amount of Trypticase (0.5g/liter) was added to the medium, the bacterium grew to high optical densities, even after repeated transfers. Strain SR grew faster with Trypticase than with Casamino acids. It did not show synergism in ammonia production when co-incubated with *Streptococcus bovis* JB1, *B. ruminicola* B₁₄ or *Selenomonas ruminantium* HD₄ in batch cultures containing Trypticase. But coculture with *S. bovis* produced 24mM ammonia.

When ruminal fluid was serially diluted and incubated with 15g l^{-1} Trypticase on four different days, the most probable number of short rods resembling strain SR was 0.75×10^7 . If mixed carbohydrates were added, the most probable number was 2.0×10^9 . As strain SR grew faster on Trypticase than on Casamino acids this suggested that short peptides were transported better than amino acids. However when gelatin hydrolysate, which is rich in large peptides, was provided little ammonia production was observed. Co-cultures of SR and peptidase producing ruminal bacteria produced much more ammonia, therefore it appears that strain SR is unable to hydrolyse long peptides extracellularly. When strain SR was grown in continuous culture at a dilution rate of 0.1 h^{-1} and the pH decreased by adding concentrated HCl to the medium, ammonia and bacterial protein in the culture decreased. At pH 5.9, strain SR washed out of the culture vessel. Strain SR is Gram variable and its taxonomy was unclear. Certain genera in the family *Bacteroidaceae* (which is a group of Gram negative, obligately anaerobic, non-spore forming, motile or non-motile rods) are Gram- variable or have Gram-positive cell wall structure. On the basis of the absence of succinate or lactate production, motility, inability to ferment carbohydrates, and fermentation products, strain SR did not resemble the genera *Bacteroides*, *Leptotrichia*, *Fusobacterium*, *Butyrivibrio*, *Succinomonas*, *Selenomonas*, or *Lachnospira*. Spores and heat stability could not be demonstrated, but these characteristics are insufficient to exclude it from the genus *Clostridium*. Strain SR produced propionate, which is atypical of the genus *Eubacterium*.

Strain F was obtained from small circular, convex, grey shiny, translucent colonies. It had a maximum specific growth rate of 0.22h^{-1} when grown on Trypticase, but when Casamino acids were added the growth was even higher (0.43 h^{-1}). Fermentation products of batch cultures containing 15g l^{-1} of Casamino acids were 29.0mM ammonia, 21.0mM acetate and 10.7mM butyrate. There was hardly any increase in the optical density when carbohydrates were added to media containing small amounts of Casamino acids. Strain F did not hydrolyze gelatin or digest casein. It produced hydrogen sulphide and indole but it did not produce any catalase, urease, lipase, or lecithinase activity. Growth was observed at 39°C but not at 25 or 45°C . There was no growth under aerobic conditions. The cells were non-motile, stained Gram positive and were sensitive to monensin ($5\mu\text{M}$). Spores were not seen

and it had a G+C content of 52.5%. It deaminated glutamine, histidine, glutamate and serine to produce ammonia at rates of 632, 200, 171, and 137 nmol /mg of protein per min respectively. When strain F was inoculated into medium containing glutamine, histidine, glutamate and serine at the same concentration as in 15g of Casamino acids per liter, the growth rate and ammonia production were similar to those observed with Casamino acids. Casamino acids provided a better energy source than Trypticase even though both had the same amino acid composition. When Strain F was co-incubated with *S. bovis* JB1 or *B.ruminicola* B₁₄, there was more than an additive increase in ammonia production. However there was synergism between this strain and *S. ruminantium* HD₄. It showed some increase in ammonia production when co-cultured with *B .ruminicola* B₁₄. When ruminal fluid was serially diluted and incubated with 15g l⁻¹ Trypticase on four different days, the most probable number of irregularly shaped organisms similar to strain F was 0.5 x10⁸. Compared with strain SR, strain F was much more resistant to low pH, and there was little decline in ammonia production or bacterial protein until the pH was less than 5.3. It is not uncommon for the ruminal pH of fattening beef cattle to be as low as 5.5 when large amounts of cereal grains are included in the ration. This often results in low ammonia production, which may be due to the more sensitive SR strains getting washed out of rumen when pH falls below 5.5. Strain F was present in the ruminal fluid in larger numbers than SR even though the pH was never below 6.3. It may be more successful as it is able to deaminate glutamine and glutamate, which are normally found in the feed stuff proteins.

On the basis of its Gram stain, morphology, inability to grow under aerobic conditions, fermentation products and its G+C content strain F had many characteristics common to the genus *Eubacterium*. *E. siraeum* produces butyrate and acetate but no *iso*-valerate or acid from glucose. However, ^{this} bacterium grew at 45 °C, did not produce indole, grew poorly in peptone-yeast broth without carbohydrates and produced large amounts of hydrogen from cellobiose, fructose, and starch. The only *Eubacterium* species to produce indole are *Eubacterium tenue*, and *E. saburreum*. The latter utilises carbohydrates and produces hydrogen, whereas the former grows on glucose and does not produce butyrate. Due to these differences, strain F could not be easily assigned to an existing species in the genus *Eubacterium*.

2.5 Phylogeny of the ammonia hyper-producing ruminal bacteria

Further study by Paster et al., (1993) focused on the phylogeny of these recently isolated strains based on 16S rRNA sequence analyses. They sequenced 95% of the 16S rRNA genes for strains C, SR and F^T and the type strain *Peptostreptococcus anaerobius*. On the basis of the sequence data, strain SR was found to be essentially identical (99.9%) to *Clostridium sticklandii*. The small differences in sequence were attributed to strain variation or sequence error. According to Bergy's Manual of Systematic Bacteriology, the biochemical traits of *Clostridium sticklandii* are in agreement with those observed for strain SR (Chen and Russell, 1989). It was also verified that strain SR was *Clostridium sticklandii* on the basis of its phenotypic reactions, its cellular fatty acid content, and the electrophoretic patterns of its cellular proteins. This was the first report of *C. sticklandii* being isolated from the bovine rumen.

The polyacrylamide gel electrophoresis protein pattern, the cellular fatty acids, and certain physiological characteristics of strain C differed from the characteristics of the type strain of *Peptostreptococcus anaerobius* (strain ATCC 27237). However strain C showed 99.6% 16S rRNA sequence similarity to *P. anaerobius*. On the basis of sequence similarity, it was suggested that strain C should be classified as a strain of *P. anaerobius*.

The taxonomic placement of strain F^T based on its phenotypic data was unclear. However its sequence data indicated that this strain required designation as a separate species in the genus *Clostridium*. Its closest relative was *C. coccoides* with 90.6% similarity. The G+C content of strain F^T was 52.5%, which is considered high for most clostridia. Since it grew rapidly with free amino acids it was named *Clostridium aminophilum*.

2.6 Enumeration of ammonia-hyper producing bacteria in the rumen

Most probable number (MPN) estimates indicated that the newly isolated strains accounted for less than 10% of the total ruminal bacterial count (Yang and Russell, 1993). Since the feed additive monensin decreased ammonia accumulation in the rumen by 50%, it was evident that a small population of obligate, amino acid- fermenting bacteria was

deaminating a large portion of the degradable protein in the rumen. However these obligate amino acid-fermenting bacteria can not be cultured in the laboratory and were not the only monensin-sensitive bacteria in the rumen (Russell and Strobel, 1989). When monensin was included as feed additive amino acid deamination decreased both *in vivo* (Dinius et al., 1976) and *in vitro* (Van Nevel and Demeyer, 1977; Russell and Martin, 1984). The obligate amino acid-fermenting bacteria were sensitive to monensin but there were carbohydrate -fermenting bacteria in the rumen, which deaminated amino acids as well. Even though the most probable number of the obligate amino acid- fermenting bacteria declined 10-fold following monensin feeding, it did not give information regarding the relative numbers of these obligate amino acid -fermenting bacteria.

16S rRNA analysis can be used to differentiate bacteria without *in vitro* culturing (Stahl et al., 1988). Krause and Russell (1996) used 16S rRNA probes to assess the contribution of obligate amino acid-fermenting bacteria *in vitro* and *in vivo*. When the 16S rRNA sequences of *Clostridium aminophilum*, *Peptostreptococcus anaerobius* and *Clostridium* were compared with sequences obtained from the Ribosomal Database Project it was possible to identify highly specific regions. Aligned 16S rRNA sequences were obtained from the Ribosomal Database Project (Larsen et al., 1993) and were compared with their closest phylogenetic relatives. Probe sequences which were homologous to the target bacterium but which had at least 3 or 4 mismatches with their closest relatives were chosen. The probes were then tested for specificity against commonly isolated rumen bacteria and a phylogenetically diverse group of ruminal and non-ruminal bacteria. Isolated rumen bacteria and mixtures of cultivated organisms enriched from rumen contents but with unknown 16S rRNA sequences were also included to adequately represent the ruminal diversity. The probes were designated as F-996, C-72 and SR-836 and were specific for *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* respectively. Preliminary experiments showed that these probes hybridised with less than 5% of rRNA from the ruminal fluid. *In vivo* studies by Krause and Russell (1996) indicated that the decrease in ammonia production following monensin feeding was correlated with a decline in the amount of 16S rRNA that hybridized with probes for *P. anaerobius*, and *C. sticklandii*. Monensin was not able to inhibit the activity of *C. aminophilum*. This bacterium does not have a large proton motive force

across its cell membrane (Russell, 1983), but it has a monensin-sensitive sodium gradient that is needed for amino acid transport and ATP formation (Russell, 1983). As monensin can bind to monensin-resistant rumen bacteria and feed particles (Chow and Russell, 1994), it may be possible that the *in vivo* monensin concentration is lower than the dose predicted solely by intake and ruminal volume. *In vitro* continuous culture experiments likewise indicated that monensin did not inhibit *C. aminophilum*. But this resistance was observed only in low-dilution rate continuous cultures that mimicked the rumen. This was not seen in batch cultures and appears to be a growth-rate dependent phenomenon.

The *in vitro* continuous culture experiments indicated that when Trypticase was added to the culture, the predominant rumen bacteria converted some of the Trypticase to ammonia but ammonia production increased significantly when *C. aminophilum*, *P. anaerobius* and *C. sticklandii* were added. These obligate amino acid fermenting bacteria accounted for less than 5% of the total 16S rRNA in continuous culture, and this result was consistent with the observation that *C. aminophilum*, *P. anaerobius* and *C. sticklandii* are found in low numbers in the rumen but have very high specific activities of ammonia production compared to other rumen bacteria (Yang and Russell, 1993).

Eschenlauer (1994) sampled cattle and sheep and enumerated numbers of monensin-sensitive, Trypticase degraders. Their viable counts indicated that bacteria capable of growth on Trypticase alone were present at 0.7% of the total bacterial population, numbers similar to those reported by Yang and Russell (1993). Like the bacteria isolated at Cornell, by Russell and his colleagues (Russell et al., 1988; Chen and Russell, 1989a) these bacteria were monensin-sensitive. However unlike the Cornell isolates, most of these bacteria fermented sugars. Ammonia production rates in the ruminal fluid from which they were derived were much lower than in the Cornell studies. It was concluded that it was not necessary to invoke the intervention of high activity bacteria. This may also be true in other studies where the rates of ammonia production are relatively low. Ammonia production in the ruminal fluid of these same animals studied by Eschenlauer (1994) was inhibited less than half by monensin, similar to the inhibitions observed in sheep fed monensin or tetronasin (Wallace et al., 1990b). Yang and Russell (1993) also found that the rate of ammonia production from casein was inhibited less than half by extremely high

monensin concentrations of 5 nmol/litre which corresponded to a likely concentration *in vivo* of 4 µmol/liter (Wallace et al., 1981) although the effect was greater for lower rates of ammonia production. Decreased ammonia production with ionophores may indeed be due to partial elimination of high activity bacteria. It should be however recognized that the influence of ionophores extends beyond those species whose growth is inhibited and are generally recognised to be monensin-sensitive. The deaminase activity of *P. ruminicola* and *Ruminobacter amylophilus* bacteria which could grow in the presence of ionophores, was greatly diminished when these were grown with ionophores in the medium (Newbold et al., 1990). Deamination of some amino acids was also affected indirectly by monensin, via decreased hydrogenase activity (Hino and Russell, 1985; Russell and Martin, 1984). The long-term effect of ionophores on ammonia reduction *in vivo* was therefore due partly to an effect on the remaining apparently ionophore-insensitive species as well as to the suppression of ionophore-sensitive, high activity species. Wallace (1996) concluded that two distinct bacterial populations carried out amino acid deamination, those with low activity but high numbers, and a second group of bacteria with high activity, but in low numbers. Wallace (1996) remarked that the former population was probably of greater significance under most circumstances. He also stated that it was extremely important to suppress the proliferation of the high activity species as their presence as a small portion of the population could have a major impact on the efficiency of N retention by the animal.

2.7 Screening Of New Zealand ruminants for HAP bacteria

The first screening of forage-fed dairy cows, sheep and deer for ammonia hyper-producing bacteria (HAP) in New Zealand was conducted by Attwood et al., (1998). They used a medium containing Trypticase and Casamino acids as the sole nitrogen and carbon source to estimate the populations of obligate peptide- and amino acid-fermenting bacteria from rumen samples. Their preliminary studies showed high levels of HAP bacteria were able to grow on this medium from these animals. Numbers of bacteria per ml of rumen fluid on HAP medium ranged from $4.99 \pm 0.64 \times 10^9$ for cows; $7.09 \pm 3.68 \times 10^8$ for deer and $1.56 \pm 0.21 \times 10^9$ for sheep. Characterization of bacteria from 14 morphologically distinct colonies indicated that there were two distinct types of organisms present; isolates C2, D1, D4, D5 and S1 which grew well on HAP medium plates and broth, and the remaining

isolates which grew poorly on plates and could not be transferred to broth cultures. Isolates C2, D1, D4, D5 and S1 appeared to be obligate peptide and amino acid fermentors as they were able to use few, if any, of the commonly fermented carbon sources. These isolates were monensin sensitive although D4 had weak growth on 5µM monensin. Three of the five isolates were Gram negative. Growth, biochemical and phylogenetic data showed that these HAP isolates were distinct from the previously described rumen HAP bacteria. Isolate D1 belonged to the genus *Peptostreptococcus* and shared many of the characteristics of *P. anaerobius*. 16S rRNA sequencing showed that although it is closely related to *P. anaerobius* (99.0% similarity) D1 has an extra 155bp in its helix 6 region and probably represents a new *Peptostreptococcus* species. Isolate S1 is a Gram-negative, anaerobic coccus growing on lactate but weakly on pyruvate and no other carbon sources. 16S rDNA sequence analysis places S1 closest to the Gram positive *P. asaccharolyticus*. Isolates D4 and D5 are Gram negative non-motile, non-spore forming rods. Their growth and biochemical characteristics place them in the family *Bacteroidaceae*, where they closely resemble the description of the genus *Fusobacterium*. The phylogenetic analysis of D4 shows 99.2% similarity to *F. necrophorum*. Isolate C2 is non-motile, non-spore forming, Gram positive, slightly curved rod. It grows very slowly on HAP medium and only pyruvate was fermented very weakly. 16S rDNA sequence indicates that its closest relative is *Eubacterium* sp. strain SC87K, but its percentage similarity of 94.4 % indicates a distant relationship. Hence C2 is described as *Eubacterium* sp.

The preferred form of nitrogen for growth of D1, D4, D5, and C2 was Tryptone, suggesting that these organisms utilized peptides more efficiently than the corresponding amino acids. Thus these organisms resembled *C. sticklandii* which grew faster on Trypticase than on Casamino acids (Chen and Russell, 1989a). Isolate S1 utilised Casamino acid and Tryptone equally and is similar to *C. aminophilum* and *P. anaerobius* (Chen and Russell, 1988; 1989a). These isolates had high specific activities of ammonia production, comparable with those reported for *C. aminophilum*, *C. sticklandii*, and *P. anaerobius*. Isolates D1 and D4 grew well and produced ammonia up to a concentration of 60mM in batch cultures. C2 and D5 produced less ammonia, but their specific activities of ammonia production were still higher than those reported for common rumen bacteria in terms of their bacterial

concentration in the culture media (Russell et al., 1988). Isolate S1 grew rapidly in HAP medium but produced only 13mM ammonia after 24 hours of growth. As it produced equimolar proportions of ammonia and acetate, it may be similar to *Acidaminococcus fermentans* in fermenting glutamate via the hydroxyglutarate pathway (Buckel and Barker, 1974).

The study of Attwood et al., 1998, has also shown that *C. aminophilum*, *C. sticklandii*, and *P. anaerobius* are not present in significant levels in New Zealand ruminants. It was thought that the high protein content and low carbohydrate content of the New Zealand pasture and the semi-continuous grazing pattern followed by the New Zealand farmers may have favoured the proliferation of the distinct group of HAP bacteria. These bacteria may be responsible for the rapid and extreme degradation of the peptides and amino acids released from pasture protein in the rumen, leading to excessive production of ammonia, and its eventual loss as urea.

The study of the HAP bacteria in New Zealand has been hampered by the non-availability of completely selective media for the isolation of these bacteria. It was difficult to obtain an estimate of the population of each of the HAP isolates in the rumen under New Zealand conditions. The present study is aimed at designing specific rDNA probes against each of these HAP bacterial isolates. These probes will enable quantitation of these bacteria, and allow the contribution of each isolate to ammonia production in pasture-grazed New Zealand ruminants to be estimated.

Chapter 3. Materials and Methods

3.1. Bacterial strains

The bacterial strains used are listed in the Table 3.1.1.

Table 3.1.1. Bacterial strains.

Bacterium	Strain	Source
<i>Butyrivibrio fibrisolvens</i>	D1 ^T	Michael Cotta, USDA Peoria, Illinois
<i>Butyrivibrio fibrisolvens</i>	H17c	Rod Mackie, University of Illinois
<i>Clostridium aminophilum</i>	F	J.B.Russell, Cornell University
<i>Clostridium clostridiforme</i>	ATCC 25537 & 29084	ATCC
<i>Clostridium proteoclasticum</i>	B316 ^T , ATCC 51982	Attwood & Reilly, 1995
<i>Clostridium sticklandii</i>	SR	J.B.Russell, Cornell University
<i>Enterococcus faecalis</i>	NCTC 775	S.Flint, New Zealand Dairy Research Institute laboratory strain
<i>Escherichia coli</i>	DH5α	GIBCO BRL
<i>Eubacterium cellulosolvens</i>	ATCC 5494	Rod Mackie, University of Illinois
<i>Eubacterium limosum</i>	ATCC 8486	“
<i>Eubacterium ruminantium</i>	SF-50	“
<i>Eubacterium tardum</i>	NCTC 12941	William Wade, Guy’s Hospital, London
<i>Fibrobacter succinogenes</i>	AC 3	Rod Mackie, University of Illinois
<i>Fusobacterium necrophorum</i>	FnS-1 NZRCC 1109, NCTC 10575	NZ Reference Culture Collection ESR Porirua
<i>Lachnospira multiparus</i>	ATCC 19307	Rod Mackie, University of Illinois
<i>Megasphaera elsdenii</i>	T81	“
<i>Peptostreptococcus anaerobius</i>	ATCC 27337	J.B.Russell, Cornell University
<i>Peptostreptococcus productus</i>	SF50	Rod Mackie, University of Illinois
<i>Peptostreptococcus asaccharolyticus</i>	NZRCC 2903 ^T , ATCC 14963	NZ Reference Culture Collection ESR Porirua
<i>Prevotella sp.</i>	C21a	Attwood & Reilly, 1995
<i>Prevotella brevis</i>	B ₁ 4	Rod Mackie, University of Illinois
<i>Prevotella ruminicola</i>	23	“
<i>Prevotella albensis</i>	M384	Harry Flint, Rowett Research Institute, Aberdeen
<i>Ruminococcus albus</i>	8	Rod Mackie, University of Illinois
<i>Ruminococcus flavefaciens</i>	FD1	“
<i>Selenomonas ruminantium</i>	HD ₄ (ATCC 12561)	“
<i>Streptococcus bovis</i>	JB1	“
<i>Streptococcus bovis</i>	B315	Attwood & Reilly, 1995.
<i>Streptococcus constellatus</i>	ATCC 27823	Chris Sissons, Wellington School of Medicine, Otago University
<i>Streptococcus equinus</i>	NCTC 10386	Athol Klieve, Queensland Dept Primary Industry, Queensland
<i>Streptococcus intermedius</i>	AR3	“
<i>Streptococcus mutans</i>	ATCC 25175	Chris Sissons, Wellington School of Medicine, Otago University
<i>Streptococcus salivarius</i>	ATCC 7013	“
<i>Succinomonas amyloletica</i>	ATCC 19206	Rod Mackie, University of Illinois
<i>Succinovibrio dextrinosolvens</i>	ATCC 27209	“
HAP isolate	C2	Attwood et al., 1998
HAP isolate	D1	“
HAP isolate	D4	“
HAP isolate	D5	“
HAP isolate	S1	“

3.2. Chemicals

Chemicals used for media preparation were of Reagent grade. Those used for buffers and reagents for molecular biology techniques were Analar grade or higher.

3.3. Media

The media used for bacterial growth are listed below:

3.3.1. HAP (hyper ammonia-producing) Medium

Components per litre:

Salt Solution 1	200ml
Salt Solution 2	200ml
Tryptone	15g
Casamino acids	15g
Na ₂ CO ₃	4g
Microminerals	10ml
Resazurin	1ml
Cysteine-HCl	0.6g
Vitamin solution	0.1ml/10ml added after autoclaving
Distilled H ₂ O	up to 1 litre

Preparation of HAP media:

All ingredients were added except cysteine-HCl and vitamins, brought to the boil in a microwave oven and cooled under a flow of oxygen-free CO₂. Cysteine-HCl was added and the medium dispensed into Hungate tubes or into serum bottles and autoclaved. Vitamins were added just before use.

For agar slants, 5ml HAP medium was added to 0.08 g of agar in Hungate tubes under CO₂ flow. The tubes were autoclaved, cooled and vitamins added before tubes were slanted to allow the agar to set.

3.3.2 Salt Solution 1 (for HAP medium)

Components per litre:

K_2HPO_4	1.46g
------------	-------

Preparation of Salt Solution 1:

Mixed to dissolve and stored in a Schott bottle.

3.3.3. Salt Solution 2 (for HAP medium)

Components per litre:

KH_2PO_4	1.46g
Na_2SO_4	2.4g
$NaCl$	2.4g
$MgSO_4 \cdot 7H_2O$	0.5g
$CaCl_2 \cdot 2H_2O$	0.02g

Preparation of Salt Solution 2:

Mixed to dissolve and stored in a Schott bottle.

3.3.4. Vitamin Solution (for HAP medium)

Components per litre:

Pyridoxamine 2HCl	200mg
Riboflavin (vitamin B ₂)	200mg
Thiamine HCl (vitamin B ₁)	200mg
Nicotinamide (Niacinamide)	200mg
Calcium panthenate (Pantothenic acid)	200mg
Lipoic acid (DL-6,8-Thioctic acid)	100mg
p Amino benzoic acid	10mg
Folic acid	5mg
Biotin	5mg
Coenzyme B ₁₂	5mg

Preparation of Vitamin solution:

Components were combined and dissolved in anaerobic distilled water (i.e. boiled and cooled under CO₂) before being filter-sterilized through a 0.22µm filter into sterile CO₂-filled serum bottles. The working stock was kept at 4°C and the remaining bottles were frozen at -20°C and thawed when required. An amount of 0.1ml was added via syringe per 9.9ml of media after autoclaving and immediately before inoculation.

3.3.5. Microminerals (for HAP medium)***Components per litre:***

Na ₄ EDTA	500mg
FeSO ₄ .7H ₂ O	200mg
MnCl ₂ .4H ₂ O	200mg
ZnSO ₄ .7H ₂ O	10mg
H ₃ BO ₃	30mg
CoCl ₂ .6H ₂ O	20mg
CuCl ₂ .2H ₂ O	1 mg
NiCl ₂ .6H ₂ O	2mg
NaMoO ₄ .2H ₂ O	3mg

Preparation of Microminerals:

Components were combined, dissolved in distilled H₂O, autoclaved in a Schott bottle and stored at room temperature. This solution was added to HAP medium before dispensing and autoclaving.

3.3.6. Complete Carbohydrate (CC) medium (Leedle and Hespell, 1980)

Components per litre:

Centrifuged rumen fluid	160 ml
Tryptone	2.0 g
Yeast extract	0.5 g
Soluble starch	0.5 g
Cellulose	0.5 g
Xylan	0.5 g
Pectin	0.5 g
Glycerine	0.5 g
Xylose	0.5 g
Glucose	0.5 g
Maltose	0.5 g
Cellobiose	0.5 g
Na ₂ CO ₃ (8% wt/vol)	50 ml
Mineral solution 1	40 ml
Mineral solution 2	40 ml
Volatile fatty acid mix	10 ml
Haemin (1mg/ml)	1 ml
Cysteine-Na ₂ S-reducing agent	0.2 ml/10 ml added after autoclaving.
Resazurin (10mg/ml)	1ml
Distilled water	up to 1 litre

3.3.7. Mineral solution 1 (for CC medium)

Components per 100ml

K ₂ HPO ₄	0.6g
---------------------------------	------

3.3.8 Mineral solution 2 (for CC medium)

Components per 100ml

KH ₂ PO ₄	0.6g
(NH ₄) ₂ SO ₄	0.6g
NaCl	1.2g
MgSO ₄ ·7H ₂ O	0.255g
CaCl ₂ ·2H ₂ O	0.169g

3.3.9. Volatile fatty acid solution (for CC medium)

Components per 100ml

Acetic acid	17ml
Propionic acid	6ml
<i>n</i> -butyric acid	4ml
<i>n</i> -valeric acid	1ml
<i>iso</i> -valeric acid	1ml
<i>iso</i> -butyric acid	1ml
<i>DL</i> -2-methylbutyric acid	1ml

3.3.10. Cysteine Sulphide solution (for CC medium)

Components per 100ml

L-cysteine HCl	2.5g
Na ₂ S·9H ₂ O	2.5g

Preparation of CC medium

All items except the reducing agent and Na₂CO₃ were added together and the media brought to boil, cooled to room temperature under CO₂ flow and the pH adjusted to 6.5. Na₂CO₃ was added and the medium dispensed anaerobically in 9.8ml aliquots into Hungate tubes. Reducing agent, 0.2ml per 9.8ml of the medium, was added via a syringe before use.

3.3.11. Lab M fastidious anaerobe agar for *Eubacterium tardum*

4.6 g of Lab M agar powder was weighed and dissolved in 100ml of distilled water. The pH was checked, (7.2) and the medium brought to boil. The medium was autoclaved at 121°C for 15 min, and allowed to cool before pouring plates .

3.3.12. Brain Heart infusion (BHI) medium for *Eubacterium tardum*

Components per 100ml:

Brain heart infusion	3.7 g
Cysteine-HCl	0.5g

Preparation of BHI medium

BHI was dissolved in distilled water and brought to boiling point and allowed to cool under CO₂ flow through the medium. The pH was adjusted to 7.0 and the medium was dispensed in 10ml aliquots into Hungate tubes.

3.3.13. PYG (peptone, yeast extract and glucose) medium for *Eubacterium tardum*

Components per 100ml:

Peptone	1g
Yeast extract	1g
Resazurin	0.4ml
Salt solution	4.0 ml
Cysteine-HCl	0.05g
Glucose	1g

3.3.14. Salt solution (for PYG medium)

Components per 100ml

CaCl ₂	0.02g
MgSO ₄	0.02g
K ₂ HPO ₄	0.1g
KH ₂ PO ₄	0.1g
NaHCO ₃	1.0g
NaCl	0.2g

Preparation of PYG medium

4ml of the Salt solution and 1g of glucose were added to the PYG medium. The medium was gently brought to boiling point and maintained at a boil for 2 minutes. The medium was cooled by keeping the flask in ice while under CO₂ flow. Cysteine-HCl was added to

the medium and then the medium was dispensed in 10ml aliquots into Hungate tubes under CO₂ flow. The tubes were then autoclaved.

3.3.15. SOB Medium

Components per litre

Yeast extract	5.0 g
Bacto Tryptone (Difco)	20 g
NaCl	0.5 g
250mM KCl	10 ml
2M MgCl ₂	5.0 ml

Preparation of SOB medium

The volume was adjusted to 1 litre and autoclaved. Sterile MgCl₂ was added just before use. SOB agar contained 1.5 % (wt/vol) agar.

3.3.16. SOC medium

SOC medium is the same as SOB medium, except after autoclaving 2ml of sterile 1M glucose solution was added.

3.3.17. LB Broth

Components per 100ml

Tryptone	1.0g
Yeast	0.5g
NaCl	1.0

Preparation of LB broth

3 ml aliquots of the LB broth were autoclaved and 3µl ampicillin (50mg/ml in 1M Tris-HCl buffer pH 7.0, filter sterilised)was added aseptically to each aliquot.

3.4. Buffers and solutions

3.4.1. Gel-loading Buffer

Components per 100ml

Bromophenol Blue	0.25g
Xylene Cyanol	0.25g
Ficoll(type 400)	25g

Reagents were combined, dissolved and stored in the refrigerator.

3.4.2. TAE Buffer

Components per 2 litre

Tris Base	96.8g
EDTA 0.5M (pH 8.0)	40ml
Glacial acetic acid	23 ml

3.4.3. TE Buffer

Components per 100ml

Tris.HCl 0.12g (10mM final concentration)	
M EDTA (pH 8.0)	0.037g

3.4.4. GTE Buffer (pH 8.0).

Components per 100ml

50 mM glucose	0.9g
25 mM Tris. HCl (pH 8.0)	0.3g
10 mM EDTA (pH 8.0)	0.37g

3.4.5. Saline-EDTA

Components per 100ml

0.15M NaCl	8.0g
0.1M EDTA (pH 8.0)	3.7g

3.4.6. Mineral salts Buffer

Components per 100ml

Mineral solution 1 (see 2.3.7)	4ml
Mineral solution 2 (see 2.3.8)	4ml
Volatile fatty acid solution (see 2.3.9)	1ml
Na ₂ CO ₃	0.4 g
Cysteine-sulphide solution (see 2.3.10)	2ml

3.5. Bacterial culturing

HAP bacterial stocks frozen on HAP slopes at -85 °C were thawed, and a portion of the thawed liquid released from the slope containing bacterial cells was inoculated into Hungate tubes containing 10ml HAP medium. These cultures were allowed to grow overnight at 38 °C.

3.6. Rumen sampling

Rumen samples were collected initially from eight fistulated Romney wethers sheep fed dried lucerne in an animal trial at AgResearch, Grasslands, Palmerston North. Rumen samples (approximately 150g) were collected before treatment and after 6, 12, 24, 48, 72, 96, 120, 144, and 168hrs of infusion with monensin or a control infusion of 0.01% (vol/vol) Triton X100. Rumen samples were collected from dorsal, ventral, anterior and posterior sites within the rumen, placed in Schott bottles and immediately taken to the laboratory. Sample pH was recorded and 100g of each sample was mixed with 100ml of anaerobic mineral salts (MS) buffer and blended for 1min in a Waring blender under a CO₂ atmosphere. One ml aliquots of the blended sample was transferred into microfuge tubes, labeled, frozen and stored at -85°C until DNA extraction.

3.7. Phenol/chloroform/isoamyl alcohol extractions

Buffer-saturated phenol (pH 8.0), chloroform and isoamyl alcohol were combined in a 25:24:1 (vol/vol/vol) ratio and an equal volume of this mixture was added to the solution to be extracted. The solution was mixed vigorously using a cyclo mixer, centrifuged at 10,000 x g for 5 minutes, and the aqueous phase removed to a fresh tube. Extractions were repeated until the organic/aqueous interphase was free of protein. The aqueous phase was finally extracted with an equal volume of chloroform/isoamyl alcohol (24:1).

3.8. Ethanol precipitation

DNA in solution was precipitated by adding 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol, and incubating at -20°C for at least an hour. Precipitated DNA was recovered by centrifugation at $12,000 \times g$ at 4°C for 20 min. The supernatant was removed and the pellet washed with 70% ethanol. After centrifugation the supernatant was removed and the pellet vacuum dried.

3.9. DNA extraction

Two different methods of DNA extraction were used.

3.9.1. Enzymatic lysis (Saito and Miura, 1963)

Bacterial cultures grown overnight were transferred aseptically to sterile centrifuge tubes within a biological safety cabinet. The cells were collected by centrifugation at $10,000 \times g$ at 4°C for 10 min. Culture supernatant was removed and the bacterial pellet was resuspended in 1ml saline-EDTA with vigorous shaking to resuspend the pellet. The washed cells were recentrifuged at $10,000 \times g$ for 5 min and the pellet was resuspended in 2ml saline-EDTA. Lysozyme (Boehringer Mannheim, Germany; final concentration, 1mg ml^{-1}) and RNase A (Life Technologies, Auckland, NZ; final concentration $20\mu\text{g ml}^{-1}$) were added and incubated at 37°C for 2.5 hr or until lysis had occurred. Sodium dodecyl sulphate (SDS, 1% wt/vol) and proteinase K (Life Technologies, Auckland, NZ; final concentration 0.2 mg ml^{-1}) were added and the mixture incubated at 65°C for 2.5 hr. The lysate was phenol/chloroform/isoamyl alcohol extracted and the DNA was precipitated from the aqueous phase with ethanol.

3.9.2. Physical Disruption (Stahl et al. 1988)

Physical disruption was used to extract DNA from rumen samples and from cells for sensitivity experiments and standard curve construction. One ml of homogenized rumen contents was added to 1.2 g of sterile Zirconia/silica beads (Biospec Products Ltd, Bartlesville, OK, USA) and spun at $10,000 \times g$ in a bench centrifuge for 1 min to pellet cells. The upper liquid portion was removed and 1ml saline-EDTA was added. The contents were shaken well to resuspend the pelleted cells and centrifuged again at $10,000 \times g$ for 1 min. The supernatant was removed, 500 μl saline-EDTA, 50 μl 20% SDS and 500 μl of Tris-equilibrated phenol (pH 8.0) were added, the contents mixed and beaten in a Mini

beadbeater (Biospec Products Ltd, Bartlesville, OK, USA) at maximum speed for 2 min. The homogenate was then incubated at 60°C for 10 minutes, cooled to room temperature, and centrifuged at 10,000 x g for 5 min. The upper aqueous portion was mixed with 500µl Tris-equilibrated phenol and centrifuged at 10,000 x g for 5 min. The upper aqueous portion was mixed with 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 10,000 x g for 5 min. This step was repeated again. Finally chloroform was added to the top aqueous portion and centrifuged at 10,000 x g for 5 min. The upper aqueous portion was removed and ethanol precipitated. The pellet was resuspended in TE buffer and RNase A (final concentration 20µg ml⁻¹) was added and incubated at 37 °C for 60 minutes. RNase A was removed by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. The air-dried DNA pellet was resuspended in TE buffer and was stored at -20 °C until required.

3.10. DNA quantitation

DNA concentration and purity were determined spectrophotometrically by measuring A_{260/280}, using a Spectramax Microplate Spectrophotometer (Molecular Dynamics, CA, USA).

3.11. Agarose gel electrophoresis

Depending on the size of the DNA fragments, samples were analyzed in 0.8 to 1.2% (wt/vol) agarose (Boehringer Mannheim, Germany) gels in 1x TAE buffer. Where DNA was to be recovered from agarose, 1.8 % low melting point agarose (Sigma, Chemical Co., MO, USA) was used. DNA samples were mixed with 6 x Gel loading buffer, loaded into the wells and electrophoresed at 8-12V cm⁻¹ for 1-2 hr. After electrophoresis, gels were stained with a 5µg ml⁻¹ solution of ethidium bromide for 10 min and destained for 20 min in water. Stained agarose gels were viewed on a UV-transilluminator, and photographed using a Polaroid MP-4 land camera and Polaroid 667 film (St. Albans, Hertfordshire, England).

3.12. Extraction of DNA from agarose

The required DNA band was excised from a low melting point agarose gel and melted at 65°C. An equal volume of 1M Tris.HCl buffer-equilibrated phenol was added, mixed and immediately frozen at -20 °C. The frozen phenol/agarose mixture was centrifuged at

10,000 x g for 10 min at room temperature and the aqueous phase removed and re-extracted with phenol/chloroform/isoamyl alcohol. The aqueous phase was ethanol precipitated and DNA resuspended in TE or sterile distilled water.

3.13. Restriction endonuclease digestions

Restriction endonuclease digests were carried out in 20µl reaction volumes in 1.5ml microfuge tubes, using buffers supplied by the manufacturer. Reactions contained 0.5 to 1.0 µg of DNA in 17µl of distilled water, 2ul of the supplied 10x buffer, and 5-10 units of the restriction enzyme. Incubations were carried out at the recommended temperature for at least 3 hours.

3.14. Ligations

Ligations were carried out in 20µl reaction volume in 1.5 ml microfuge tubes using the 5x DNA Ligase buffer supplied by the manufacturer. Reactions contained 0.5-1.0 µg of DNA in 15µl of distilled water, 4µl of 5x DNA Ligation Buffer and 1µl containing 10 units of T4 DNA ligase (Gibco BRL, Life Technologies, Auckland, NZ). The tubes were incubated at 23 °C for 1.5 hours to facilitate ligation of cohesive termini or at 14°C for 24 hours for the ligation of non-cohesive or blunt termini. The products of ligation reactions were diluted to 100µl with sterile distilled H₂O before use in transformation or PCR reactions.

3.15. Cloning PCR products

Insertions into the vector, pGEM-T (Promega Corporation, WI, USA) were carried out in a 10µl reaction volume in 1.5ml microfuge tubes, using 2x Rapid Ligation buffer supplied by the manufacturer. Reactions contained insert DNA at a vector:insert ratio of approximately 1:1, 5µl of 2x Rapid Ligation buffer, 10 units of T4 DNA Ligase and 50ng of the pGEM-T vector. The reactions were incubated at 4°C overnight to produce maximum number of insertions.

3.16. Transformations

A modified protocol of Sambrook *et al.*, (1989) was used for the transformation of plasmid DNA into *E. coli* DH5- α competent cells (Gibco BRL, Life Technologies, Auckland, NZ). To determine transformation efficiency, pUC19 was used as positive control. Competent

cells without any added DNA was used as a negative control. Approximately 10ng of transforming DNA was added to 50 μ l of competent DH5 α *E. coli* cells. The contents of the tube were mixed gently and allowed to sit on ice for 30 min. Cells were heat-shocked at 37 °C for 20 seconds, and quickly chilled on ice for 2 min. Cells were diluted with 950 μ l of SOC medium and incubated at 37 °C with gentle shaking for 1 hr. Aliquots of transformed cells were plated onto SOB solid medium, containing ampicillin (100 μ g ml⁻¹), X-gal (50 μ g ml⁻¹) and isopropyl- β -D-thiogalactopyranoside (0.16mg ml⁻¹). Plates were incubated overnight at 37 °C.

3.17. Plasmid DNA minipreparations

Plasmid DNA was recovered from *E. coli* cells using a modified alkaline-lysis/PEG precipitation procedure method. Three ml of LB medium containing ampicillin (50 μ g ml⁻¹) was inoculated with a single colony of a plasmid-bearing *E. coli* clone and incubated at 37 °C overnight with vigorous shaking. An aliquot (1.5 ml) of culture was transferred aseptically into a microfuge tube and centrifuged in a bench-top centrifuge at 10,000 x g for 2 minutes. The supernatant was removed and the bacterial pellet was resuspended in 200 μ l GTE buffer with vigorous vortexing. Freshly prepared 0.2M NaOH / 1% SDS solution (300 μ l) was added, and the tube rapidly inverted several times before incubating on ice for 5 min. Potassium acetate, (3.0M, pH 4.8, 300 μ l), was added to neutralize the solution and the tube inverted again to mix contents and incubated on ice for 5 min. The contents were centrifuged at 12,000 x g at room temperature for 10 min and the supernatant transferred to a fresh tube. RNase A was added to a final concentration of 20 μ g/ml and the tubes were incubated in a water bath at 37 °C for 20 min. The supernatant was then extracted twice with 400 μ l of chloroform. The layers were mixed by hand, the tube centrifuged for 1 min to separate the phases and the upper aqueous phase transferred to a fresh tube. An equal volume of 100% isopropanol was added and immediately centrifuged for 10 min at room temperature to precipitate the DNA. The DNA pellet was washed with 500 μ l of 70% ethanol and dried under vacuum for 3 min. The pellet was dissolved in 36 μ l of sterile deionized water. A small aliquot (4 μ l) of the dissolved DNA was mixed with distilled water (6 μ l) and Gel Loading buffer (2 μ l) and electrophoresed through a 0.8 % agarose gel to visualize the plasmid DNA, estimate size and confirm the presence of inserts. NaCl (4M, 8.0 μ l) was then added to the remaining 32 μ l of plasmid DNA and 40 μ l of 13% PEG 8000 to precipitate the dissolved plasmid DNA. After thorough mixing the sample was

incubated on ice for 20 min and the plasmid DNA pelleted by centrifugation at 10,000 x g at 4°C for 15 min. The supernatant was carefully removed and the pellet rinsed with 500µl of 70% ethanol. The pellet was dried under vacuum for 3 min and resuspended in 20µl of deionized water and stored at -20 °C.

3.18. Preparation of HAP isolates for sensitivity testing

Cultures of HAP isolates namely C2, D1, D4, D5, S1, *P. anaerobius*, *C. aminophilum*, and *C. sticklandii* were grown in 100ml overnight cultures, and a sample from each was counted by phase-contrast microscopy using a WSI counting chamber (Weber Scientific International Ltd., Middlesex, England). The bacterial cells were pelleted by centrifugation, and DNA extractions were performed. Ten-fold serial dilutions of each of the above DNA samples were then amplified with its specific primer to determine the detection limit. Using this dilution, the DNA samples were then co-amplified with serial dilutions of the internal controls constructed for each primer to determine the dilution point at which internal control DNA and target DNA coamplified at equal intensity.

3.19. Quantitation of PCR products

PCR products were quantitated by photographing agarose gels with Polaroid 665 film (St. Albans, England), which produces a negative image of the photograph. The negative was scanned using a GS-670 imaging densitometer (BioRad, Hercules, CA, USA.) and analyzed with Molecular Analyst software version 1.4 (BioRad, Hercules, CA, USA). To correct for differences in the fluorescence of ethidium bromide-stained PCR fragments of different sizes (Piatak et al., 1993), the intensity of the internal control was multiplied by the ratio target size (bp) /internal control size (bp).

3.20. Animal Trial

An experiment was conducted using 8 sheep fitted with rumen and abomasal cannulae at AgResearch Grasslands, Palmerston North, New Zealand. 20, 1 year old Romney wether sheep with a mean live weight of 29.5kg were purchased. All sheep were treated for external parasites (10ml Wipeout; Coopers Animal Health (NZ) Ltd, Wellington, New Zealand), and drenched with anthelmintic to control internal parasites (12 ml Ivomec; Merc Sharp and Dohme (NZ) Ltd) prior to being placed in metabolism crates. After a week in metabolism crates 12 animals were selected for surgery, based on eating habits and

adaptation to the metabolism crates. Surgery involved rumen fistulation (63 mm ID) and abomasal cannulation (10 mm ID). A recovery period of 5 weeks was allowed during which the animals were fed 300gm lucerne pellets and 700gm chaffed lucerne. The sheep were then moved to indoor experimental rooms and positioned underneath automatic belt feeders and fed a diet completely of chaffed lucerne (0.9kg/day), (g/kg DM: N = 21, Total AA = 108.6, Essential AA = 52.5, Non-essential AA = 56.2). Feed was delivered every hour to feed bins over a period of 24 hours. Automatic feeders were topped up every morning at 0800 hours. Water was provided *ad libitum*. One group of sheep (n = 4) received a continuous intraruminal infusion of monensin (17mg/L) in Triton X100 buffer (0.01%) at a rate of 23mg/day/sheep, a total of 1400ml/day being infused from day 2 until slaughter on day 9.) while the control group (n=4) received a continuous intraruminal infusion of 0.01% Triton X100. A total of 1400 ml/day/sheep was given from day 4 until slaughter on day 11. Rumen sampling (see above) was conducted throughout the experiment for microbiological analysis. At the end of the experiment all sheep were euthanased by intrajugular overdose of sodium pentobarbitone (300mg/ml).

Chapter 4. Results

4.1. Introduction

16S rRNA sequence provides specific sites for probe designs at different taxonomic levels, enabling enumeration of bacterial populations in the rumen(Stahl et al., 1988; Briesacher et al; 1992; May et al., 1993; Krause and Russell, 1996). It has been widely used in the identification and phylogenetic analysis of bacteria, both for previously cultured organisms and for 16S genes cloned directly from environmental samples (Pace et al., 1986; Amann, 1995; Lee et al., 1996). It does not however represent the entire genome, but it only contains information about the 16S gene which enables inferences to be drawn from the phylogenetic analysis (Woese et al., 1975; Fox et al., 1980; Pace et al., 1986; Olsen and Woese, 1993).

Ribosomes are central to protein translation and this function is conserved among organisms. R RNAs are therefore conserved as they form the critical active site of the ribosome and their sequences can be used to study phylogenetic relationship among organisms. As rRNA presents a stem-loop secondary structure, (i.e. conserved stems and variable loops) the 16S molecule lends itself in the designing of probes at different taxonomic levels. These probes can then be used in a “nested” fashion to give information about microbial populations at various taxonomic levels. However the HAP bacteria do not seem to form a coherent phylogenetic group and are not closely related to one another. Therefore a single probe cannot encompass all HAP bacteria and this necessitates designing of probes to each HAP bacterial strain.

4.2. Results

4.2.1. 16S sequence analysis and primer design

Primers were designed for five HAP bacteria isolated from rumen samples obtained from New Zealand ruminants (Attwood et al., 1998), namely strains C2, D1, D4, D5 and S1 and three previously described isolates, *Clostridium aminophilum*, *C. sticklandii* and *P. anaerobius* (Paster et al., 1993). 16S ribosomal DNA sequences for the New Zealand isolates were retrieved from the Genbank Database (accession numbers AF044945 - AF044948 for strains C2, S1, D1 and D4 respectively) and from the Rumen Microbiology Unit, AgResearch, Grasslands, Palmerston North (for strain D5). Initially the ten most closely related 16S rDNA sequences to each of the New Zealand HAP bacterial sequences were identified using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology (NCBI). The DNA sequences of the closely related organisms were aligned with the 16S rDNA sequence of each HAP isolate using the ClustalX multiple sequence alignment program (Thompson, et al. 1994). Figures 4.1 to 4.5 show the relevant part of each alignment where primers were designed. The probes for *Clostridium aminophilum*, *C. sticklandii* and *Peptostreptococcus anaerobius* were adapted for use as PCR primers from those designed by Krause and Russell (1996).

A.

C2	ACTTCGATT- - AATACCTCATAACGCATCGA	GGTCGCATGG	203
E.brachy	ACGAATATT- - AATACCTTATGAACTTA	ACTAGTGCATGC	210
E.infirmum	AAGGTGATT- - AATACCCCATGATGCAAGAGATACACATGT		183
E.minutum	ACTACGATT- - AATACCTCATAAAGCAAAAGCTTCGCATGG		204
E.minutum2	ACTACGATT- - AATACCTCATAAAGCAAAAGCTTCGCATGG		206
E.nodatum	ATTTTCGATT- T AATACTTCATAAAGCAGAGCATTCGCATGG		205
E.sp(sk8)	ACTACGATTT - AATACCTCATAAAGCAAAAGCTTCGCATGG		217
E.tardum	ACTACGATT- - AATACCTCATAAAGCAAAAGCTTCGCATGG		184
F.sulci	ACTACGATTT - AATACCTCATAAAGCAAAAGCTTCGCATGG		217

B.

C2	AGGCGACGGAGCGATCCTGAGCGA	ACCCCAAAG	1269
E.brachy	AAGCAAGACCGCAAGGTGGAGCAAAGCTCA	AAAAA	1278
E.infirmum	CAGCGAACCCGTGAGGGGGAGCGAATCCCA	AAAAG	1249
E.minutum	AAGCGAAAGAGCGATCTTAAGCCAAACCA	AAAAAG	1251
E.minutum2	AAGCGAAAGAGCGATCTTAAGCGAAACCA	AAAAAG	1252
E.nodatum	AAGCGAAAGAGCGATCTTAAGCCAAACCA	AAAAAG	1249
E.sp(sk8)	AAGCGAAAGAGCGATCTTAAGCCAAACCA	AAAAAG	1286
E.tardum	AAGCGAAAGAGCGATCTTAAGCCAAACCA	AAAAAG	1251
F.sulci	AAGCGAAAGAGCGATCTTAAGCCAAACCA	AAAAAG	1286

Figure 4.1. Alignments of C2 16S rDNA.

The 176bp region (A) and the 1248 region (B) of the C2 sequence are shown aligned to their most closely related sequences. Sequences were retrieved from the data base indicated and were aligned using the Clustal X alignment program. Only the regions of sequence relevant to designing the probes are shown. Accession numbers of the sequences are as follows: C2 (gb AF044945), *Eubacterium brachy* (emb Z36272), *E. infirmum* (gb U13039), *E. minutum* (emb AJ005636), *E. minutum2* (dbj AB020885), *E. nodatum* (gb U13041), *E. sp(sk8)* (emb 36275), *E. tardum* (gb U13037), *F. sulci* (emb AJ006963). The data base abbreviations are gb, Genbank; emb, European Molecular Biology Laboratory Nucleotide Sequence Database; dbj, DNA Database of Japan.

Peptostreptococcus	-----AGT-----CGGA	92
P.anaerobius	CGAGCGCGTCTNA-----TTTG	79
D1	T[CCTTGTTGATAA-GATAAAGCTTTGAGTTCTAAGGATAAGT]	204
C.paradoxum3	-----	59
C.paradoxum2	-----AG-----CATA	160
C.paradoxum	-----AG-----CATA	160
C.irregularis	-----	63
C.glycolinum	-----	54
C.bifermentan	-----	65
C.bifermentan	-----	65

Figure 4.2. Alignments of D1 16S rDNA sequence.
The 165bp region and the 185 region of the D1 sequence are shown aligned to their most closely related sequences. Sequences were retrieved from the databases indicated and aligned using the Clustal X alignment program. Only the regions of sequence relevant to designing the probes are shown. Accession numbers of the sequences are as follows:
D1 (gbAF044947), *Peptostreptococcus* sp (gb AF044947) *P. anaerobius* (gb L04168), *C. paradoxum3* (emb Z69934), *C. paradoxum2* (emb Z69931),*C. paradoxum* (emb Z69941) *C. irregularis* (emb X73447), *C. glycolinum* (emb X76750), *C. bifermentans* (emb 73437), *C.bifermentans* (emb X7590). The abbreviations used are gb, Genbank emb, European Molecular Biology Laboratory Nucleotide Sequence Database.

A.

F.gonidoformans	TGGACAATGGACCAAAAGTCTGATCCAGCAAT	388
F.gonidoformans2	TGGACAATGGACCAAAAGTCTGATCCAGCAAT	304
D4	TGGACAATGGACCACAAGTCTGATCCAGCAAT	371
F.rusii	TGGACAATGGACCAAGAGTCTGATCCAGCAAT	385
F.rusii2	TGGACAATGGACCAAGAGTCTGATCCAGCAAT	304
C.rectans	TGGACAATGGACCAAGAGTCTGATCCAGCAAT	387
F.necrophorum	TGGACAATGGACCAAGAGTCTGATCCAGCNAT	304

B

F.gonidoformans	ACAAAGAGTGCAGAGATGCGCTTGTGCTTCTTCGGAAGAA	1006
F.gonidoformans2	ACNAAGAGTGCAGAGATGCGCTNGTGCTTCTTCGGAAGAA	918
D4	ACGAACGGAGCAGAGATGCGCCGGTGCCCTTTCGGGGGAA	989
F.rusii	ACTAAGGGCTCAGAGATGAGCTTGTGCTCCTTCGGGAGAA	1001
F.rusii2	ACTAAGGGCTCAGAGATNAGCTNGTGCTC-TTCGGAAGAA	919
C.rectum	AAGAAATTAGCAGAGATGCTTTTGTGCC--TTCGGGGGAA	1007
F.necrophorum	AAGAAATNAGCAGAGATGCTNTNGTGCTC---TTCGGGGNAA	916

Figure 4.3. Alignments of D4 16S rDNA sequence. The 343bp region (A) and the 952 region (B) of the D4 sequence are shown aligned to their most closely related sequences. Sequences were retrieved from the database indicated and were aligned using the Clustal X alignment program. Only the regions of sequence relevant to designing the probes are shown. Accession numbers of the sequences are as follows: D4 (gb AF0 44948), *F. gonidoformans* (gb M58679), *F. gonidoformans2* (emb X55410), *F. rusii* (gb M58681), *F. rusii2* (emb X55409), *C. rectum* (emb X77850), *F. necrophorum* (gb AF044948). The data base abbreviations are gb, Genbank; emb, European Molecular Biology Laboratory Nucleotide Sequence Database.

A.

D5	CTCGGGTGTAAACCACTGGCTTTGGGGACG	500
R.albus1	TTAGGATTGTAAACCTCTGTCTTTGGGGACG	413
R.albusOR108	TTAGGATTGTAAACCTCTGTCTTTGGGGACG	439
R.albus2	TTAGGATTGTAAACCTCTGTCTTTGGGGACG	437
R.callidus1	TTCGGATTGTAAACTCCTGTTGAAGAGGACG	402
R.flavefaciens	TTCGGATTGTAAACTCCTGTCTTAAAGGACG	422
R.flavefaciens	TTCGGATTGTAAACTCCTGTCTTAAAGGACG	417
R.flavefaciens	TTCGGATTGTAAACTCCTGTCTTAAAGGACG	442

B.

D5	CATCTCCTGAGTAGCCTAGAGATAGGTGATC	1049
R.albus1	CATCGTACGCATAGCATAGAGATATGTGAAA	963
R.albusOR108	CATCGTACGCATAGCATAGAGATATGTGAAA	989
R.albus2	CATCGTACGCATAGCATAGAGATATGTGAAA	987
R.callidus1	CATCGTACGCATAGCATAGAGATATGTGAAA	961
R.callidus2	CATCGAGTGACGTACCTAGAGATAGGT--AT	950
R.flavefaciens1	CATCGTATGCATAGTCTAGAGATAGATGAAA	972
R.flavefaciens2	CATCGTATGCATAGCATAGAGATATGTGAAA	967
R.flavefaciens3	CATCGTATGCATAACTTAGAGATAAGTGAAA	992

Figure 4.4. Alignments of D5 16S rDNA sequence. The 477bp region (A) and the 1023bp regions (B) of the D5 sequence are shown aligned to their most closely related sequences. Sequences were retrieved from the database indicated and were aligned using the Clustal X alignment program. Only the regions of sequence relevant to designing the probes are shown. Accession numbers of the sequences are as follows: *R. albus* 1 (gb AF079847), *R. albus* OR 108 (gb AF030452), *R. albus* 2 (gb L76598), *R. callidus* 1 (emb X85100), *R. callidus* 2 (gb L76596), *R. flavefaciens* 1 (gb AF030446), *R. flavefaciens* 2 (emb X83430), *R. flavefaciens* 3 (gb L76603). The D5 16SrDNA sequence was obtained from the Rumen Microbiology Unit, AgResearch. The data base abbreviations are gb, Genbank; emb, European Molecular Biology Laboratory Nucleotide Sequence Database.

A.

S1	CAACCCTCGTAA-GCATTGAAACTTCTAA	621
Tissierella	CAACTGTGGTAA-GCACTTGAAACTGAAAG	602
Peptostreptococcus sp.	NAACCGTAGTTA-GCCTCCGAAACTGGAAG	624
P.prevotti	CAACCCATGTAA-GCATTNAAACTGGTAG	629
C.barkeri	CAACCGGNNACGTGCATTTGAAACTGTAAG	639
E.angustum	CAACCATTGCAA-GCACTTGAAACTGAAGN	636
P.assacharolyticus	CAACCCTTGTAAGCATTNAAACTAATAA	632
P.hareii	TAACTACTGTAA-GCATTGAAACTGCATA	610
P.ivoricus	TAACCTTCGYAA-GCATCGGAACTGTCGA	609

B.

S1	ACCTTACCAGGCTTGACATAATATTGACCT	973
Tissierella	ACCTTACCAGGGCTTGACATGCCAAAGACCG	958
Peptostreptococcus sp.	ACCTTACCTAAGCTTGACATCCCTTAGACCG	981
P.prevotti	ACCTTACCAAGGCTAGACATTTTAATGACCG	979
C.barkeri	ACCTTACCAGGCCTTGACATCCTCTGACCAT	991
E.angustum	ACCTTACCAAGGCTTGACATCCCTCTGACCG	992
P.assacharolyticus	ACCTTACCAGGGCTTGACATAATATTGACCG	983
P.hareii	ACCTTACCAGGGCTTGACATACTGAGGACCG	962
P.ivoricus	ACCTTACCGGACTTGAAATATTGTCGCCAA	963

Figure 4.5. Alignments of S1 16S rDNA sequence.

The 600bp region (A) and the 954bp (B) of the S1 sequence are shown aligned to their most closely related sequences. Sequences were retrieved from the database indicated and were aligned using the Clustal X alignment program. Only the regions of sequence relevant to designing the probes are shown. Accession numbers of the sequences are as follows: S1 (gb AF044946), *Tissierella* sp- (emb X80227), *Peptostreptococcus* sp. (emb X90471), *P. prevotii* (dbj D14153), *C. barkeri* (gb M23927), *Eubacterium angustum* (gb L34612), *P. asaccharolyticus* (dbj D14138), *P. hareii* (emb Y07839), *P. ivoricus* (emb Y07840). The data base abbreviations are gb, Genbank; emb, European Molecular Biology Laboratory Nucleotide Sequence Database; dbj, DNA Database of Japan.

The alignments were examined to identify unique regions within the HAP bacterium 16S sequences. Unique sites were then analysed for possible probe sites following the principles for developing oligonucleotide probes (Stahl and Amann, 1991). As the competitive PCR reactions are “anchored” at either the 5’ or 3’ end of the 16S gene using the universal eubacterial primers fd1* or rd1* respectively, the specific primers were designed to be as similar as possible to their respective “anchor” in respect to base length, %G+C and T_m . For probe sites closest to the 5’ end of the 16S gene, probes were designed directly from the sequence and used with the universal eubacterial reverse primer (rd1*) in PCR reactions. Probe sites closest to the 3’ end of the 16S gene used the reverse complement of the sequence and used the universal eubacterial forward primer (fd1*) as their primer pair. Two primers were selected for each of the 5 New Zealand HAP isolates and are listed in Table 4.1.

Strain C2 was most closely related to species of the genus *Eubacterium*. Inspection of the 16S rDNA sequence alignment showed unique sites within the C2 sequence at 191 to 195 bp (variable region V2) and 1248 bp (variable region V8). The D1 isolate was most closely related to *Peptostreptococcus anaerobius*, *Clostridium paradoxum*, *C. irregularis*, *C. glycolinum* and *C. bifermentans*. An unusual feature of strain D1 16S rDNA is that it has an additional 155 bp located within its Helix 6 region (bp positions 50 to 204, variable region V1). Two primers were designed within this region at bp position 165 to 184 and from bp position 185 to 204. Strain D4 was closely related to *Fusobacterium necrophorum* (98.8%) and more distantly related to *F. gonidiformans*, *F. rusii*, and *C. rectum*. Due to the high similarity of the D4 and *F. necrophorum* sequences it was difficult to design primers that were greatly different. One primer was designed at bp position 343 to 362 and another at bp position 952 to 961. Strain D5 was closely related to *Ruminococcus albus*, *R. flavefaciens* and *R. callidus*. One primer was designed at bp position 477 to 496 (variable region V3) and another at bp position 1023 to 1042 (variable region V6). Strain S1 was most closely related to *Peptostreptococcus asaccharolyticus* (96.9%) and less closely related to an unidentified *Tissierella* species, an unidentified *Peptostreptococcus* species and *Eubacterium angustum*. One primer was designed from bp position 600 to 619 (variable region V4) and another from bp position 954 to 973.

The candidate probes were then checked for specificity using the PROBE CHECK program of the Ribosomal Database Project (Olsen *et al.*, 1993) and screened for mismatches, insertions, deletions, or any self-complementarity within the probes. The probes were also submitted to the Basic Local Alignment Search Tool (BLAST) facility at the National Center for Biotechnology Blast to ensure specificity and to verify the most closely related bacteria.

Table 4.1: Primers

Target Organism	Primer						
	Designation	Site (bp)	Sequence (5'to 3')	Length (bp)	% G+C	T _m (°C)	Primer pair
C2	C2/176	176-195	TACCTCATAACGCATCGAGG	20	50	60	rd1*
C2	C2/1248r	1248 - 1267	TTGGGGGTTTCGCTCAGGATC	20	60	64	fd1*
D1	D1/165	165 - 184	CCTTGTTGATAAGATAAGGC	20	35	54	rd1*
D1	D1/185	185 - 204	TTTGAGTTCTAAGGATAAGT	20	30	52	rd1*
D4	D4/343	343 -362	ACAATGGACCACAAGTCTGA	20	45	58	rd1*
D4	D4/952r	952 - 971	GAACGGAGCAGAGATGCGCC	20	65	66	fd1*
D5	D5/477	477 - 496	TGTAAACCACTGGCTTTGGG	20	50	60	rd1*
D5	D5/1023r	1023-1042	TATCTCTAGGCTACTCAGGA	20	45	58	fd1*
S1	S1/600	600 - 619	CGTAAGCATTTGAAACTTCT	20	35	54	rd1*
S1	S1/954r	954 - 973	AGGTCAATATTATGTCAAGC	20	35	54	fd1*
<i>P. anaerobius</i>	C/980r	980 - 999	GGTGTGATTAAACACGGG	18	50	54	fd1*
<i>C. aminophilum</i>	F/966r	966 - 985	CTGCGACTTTTGCATCACATTCAGAT	26	42.3	74	fd1*
<i>C. sticklandii</i>	SR/836r	836 -855	GGCACCGACCTTTGACAG	18	61	58	fd1*

4.2.2. Primer optimisation

The selected primers were tested under a range of temperatures and MgCl₂ concentrations to determine optimum amplification in PCR reactions with their respective primer pairs. Table 4.2 gives the final list of the primers selected for PCR on the basis of their amplification efficiency at their optimized annealing temperature and MgCl₂ concentration.

Table 4.2. Optimized PCR conditions for primer amplification

Specific primer	Primer pair	Annealing temperature (°C)	MgCl ₂ concentration (mM)
C2-176	rdl *	50	2.0
C2-1248r	fdl *	65	2.5
D1-165	rdl *	50	2.0
D1-185	rdl *	50	2.0
D4-343	rdl *	46	2.0
D4-952r	fdl *	64	2.5
D5-477	rdl *	58	2.0
D5-1023r	fdl *	50	2.0
S1-600	rdl *	50	2.0
S1-954r	fdl *	63	2.0
C 980	fdl *	63	2.5
F 966r	fdl *	67	2.5
SR 836	fdl *	67	2.5

As the primers C2-1248r, D4-952r, D5-477, S1-954r, amplified more efficiently than C2-176, D4-343, D5-1023r, and S1-600 and were chosen for further testing. C-980r, F-966r and SR-836 were also included in these tests.

4.2.3. Primer specificity

The primers were tested for specificity by carrying out PCRs with DNA extracted from 31 selected rumen bacteria. The previously defined optimal annealing temperatures and MgCl₂ concentrations were used for each primer pair and the results are listed in Table 4.3.

The C2/1248r and D5/477 primers produced amplified PCR products only with their respective target DNAs while primers S1/954r, D4/952r and C980 amplified DNA from closely related species *P. asaccharolyticus*, *F. necrophorum* and strain D1 respectively.

Table 4.3. Primer specificity

Bacterial strain	Primer						
	C 980	SR 836	F 966	C2 1248r	D4 952r	D5 477	SI 954
<i>Streptococcus bovis</i> JB1	-	-	-	-	-	-	-
<i>Streptococcus bovis</i> B315	-	-	-	-	-	-	-
<i>Streptococcus thermophilus</i>	-	-	-	-	-	-	-
<i>Streptococcus constellatus</i>	-	-	-	-	-	-	-
<i>Streptococcus mutans</i>	-	-	-	-	-	-	-
<i>Streptococcus salivarius</i>	-	-	-	-	-	-	-
<i>Streptococcus intestinalis</i>	-	-	-	-	-	-	-
<i>Streptococcus equinus</i>	-	-	-	-	-	-	-
<i>Ruminococcus flavefaciens</i> FD1	-	-	-	-	-	-	-
<i>Ruminococcus albus</i>	-	-	-	-	-	-	-
<i>Megasphaera elsdenii</i>	-	-	-	-	-	-	-
<i>Fibrobacter succinogenes</i>	-	-	-	-	-	-	-
<i>Lachnospira multiparus</i>	-	-	-	-	-	-	-
<i>Selenomonas ruminantium</i> HD ₄	-	-	-	-	-	-	-
<i>Succinomonas amyloletica</i>	-	-	-	-	-	-	-
<i>Succinovibrio dextrinosolvens</i>	-	-	-	-	-	-	-
<i>Clostridium sticklandii</i>	-	+	-	-	-	-	-
<i>Clostridium clostridioforme</i>	-	-	-	-	-	-	-
<i>Clostridium proteoclasticum</i> B316 ^T	-	-	-	-	-	-	-
<i>Butyrivibrio fibrisolvens</i> D1	-	-	-	-	-	-	-
<i>Butyrivibrio fibrisolvens</i> H17c	-	-	-	-	-	-	-
<i>Escherichia coli</i> K12 (DH5α)	-	-	-	-	-	-	-
<i>Prevotella bryanti</i> B ₁ 4	-	-	-	-	-	-	-
<i>Prevotella brevis</i> GA33	-	-	-	-	-	-	-
<i>Prevotella ruminicola</i> 23	-	-	-	-	-	-	-
<i>Prevotella albensis</i> M384	-	-	-	-	-	-	-
<i>Eubacterium cellulosolvens</i>	-	-	-	-	-	-	-
<i>Eubacterium limosum</i>	-	-	-	-	-	-	-
<i>Eubacterium ruminantium</i>	-	-	-	-	-	-	-
<i>Eubacterium tardum</i>	-	-	-	-	-	-	-
<i>Peptostreptococcus asaccharolyticus</i>	-	-	-	-	-	-	+
<i>Peptostreptococcus anaerobius</i>	+	-	-	-	-	-	-
<i>Peptostreptococcus productus</i>	-	-	-	-	-	-	-
<i>Enterococcus faecalis</i>	-	-	-	-	-	-	-
<i>Fusobacterium necrophorum</i>	-	-	-	-	+	-	-
C2	-	-	-	+	-	-	-
D1	+	-	-	-	-	-	-
D4	-	-	-	-	+	-	-
D5	-	-	-	-	-	+	-
S1	-	-	-	-	-	-	+

4.2.4. Construction of internal controls

Internal controls for the 7 selected primers were constructed following the technique outlined for primer C2-1248r in Fig 4.6. Each HAP bacterium-specific primer was used with their respective universal primer to produce a PCR product from genomic DNA from each respective HAP bacterial isolate. The PCR product was digested using restriction endonucleases and the terminal fragments containing the specific and universal primer binding sites were religated to create a deleted form of the target DNA which can be used as an internal control (Table 4.4). A portion of the ligation reaction was used in a PCR reaction with the specific and universal primers and the PCR product corresponding to the correct predicted size was excised from the gel and purified. The purified internal control DNA was then cloned into the PCR cloning vector, pGEM-T, transformed into competent *E. coli* DH5 α cells. Transformants containing internal control DNA-bearing plasmids were selected, grown and plasmid DNA extracted. Dilutions of the cloned form of the internal control DNAs were used in competitive PCRs.

Before using the internal controls in cPCRs the reaction conditions for each of the specific primers was reoptimised in the presence of the internal control DNAs. Reoptimised conditions were exactly as determined previously except that the C2-1248r/fd1* primer pair amplified best at 64 °C (previously 65°C) and the D4-952r/fd1* primer pair amplified best at 63 °C and 2.0mM MgCl₂ (previously 64 °C and 2.5 mM MgCl₂). The reoptimised conditions for C2-1248r/fd1* and D4-952r/fd1* did not alter primer specificity as these new conditions failed to amplify DNA from their closest relatives (data not shown).

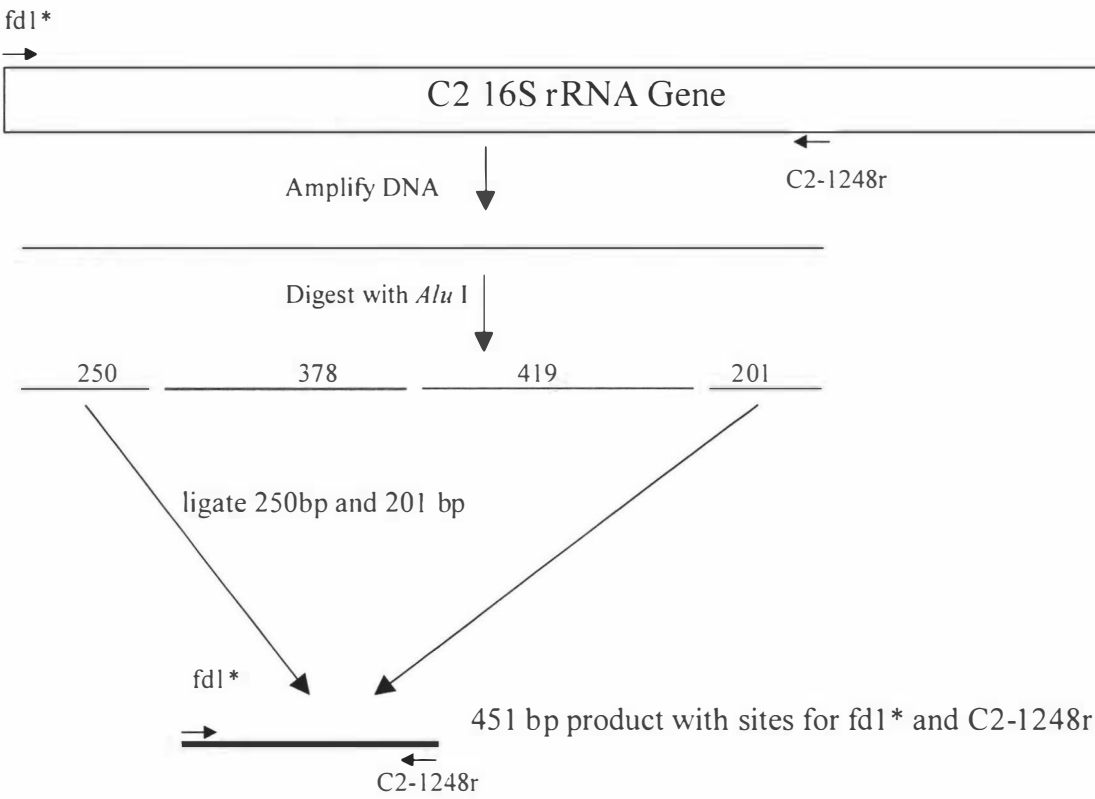


Figure 4.6. C2 internal control construction
The 1248 bp PCR product from strain C2 genomic DNA amplified with the universal fdl* and C2-1248r primer contains three *Alu* I sites at 250bp and 628bp and 1047bp. To produce an *Alu* I deletion, the 1248 bp fragment was digested with *Alu* I and the 250bp and 201bp *Alu* I fragments were religated. A portion of the ligation reaction was used in a PCR reaction with the universal fdl* and C2-1248r primers. The 451 bp PCR product containing the universal fdl* and C2-1248r priming sites was excised from the gel, purified, cloned in pGEM-T and used as internal control for competitive PCRs.

Table 4.4. Construction of internal controls

Primer pair	PCR product size (bp)	Restriction enzyme	Restriction fragment sizes (bp)	Internal control PCR product size (bp)
C2-1248/fd1*	1248	<i>Alu</i> I	250 [†] , 378, 419, 201 [†]	451
D5-477/rd1*	1023	<i>Ban</i> II	163 [†] , 311, 549 [†]	712
D4-952r/fd1*	952	<i>Alu</i> I	181 [†] , 20, 16, 349, 241, 145 [†]	326
S1-954/fd1*	954	<i>Msp</i> I	150 [†] , 138, 188, 52, 163, 263 [†]	413
SR-836/fd1*	836	<i>Hae</i> III	198 [†] , 78, 103, 457 [†]	655
F-966/fd1*	966	<i>Hae</i> III	276 [†] , 27, 13, 22, 400, 224 [†]	500
C980/fd1*	980	<i>Cfo</i> I	64 [†] , 314, 602 [†]	666

[†] terminal fragments

4.2.5. Amplification of rumen DNA with specific primers

Before attempting to quantitate bacterial populations using the cPCR technique it is necessary to first check that the rumen DNA is amplifiable using PCR and that there are detectable levels of the target organisms within the samples. Therefore DNA extracted from rumen samples before treatment, and from samples collected at 24, 48, 72 and 96 hr after treatment with monensin or from control sheep were tested with universal forward (fd1*) and reverse (rd1*) primers at an annealing temperature of 55 °C. All samples produced a PCR product of approximately 1500 bp which corresponds to the expected size of bacterial 16S rRNA genes, showing that these DNA samples are amplifiable. The samples were also amplified with each of the seven specific primers at their optimum annealing temperatures and MgCl₂ concentrations in the absence of internal control DNAs. Internal control DNAs were left out of the reactions, as they compete with target DNA and decrease the overall sensitivity of PCR (Reilly and Attwood, 1998). All the samples gave PCR products of the predicted size with the D4-952r and C-980 primers, but all samples failed to amplify with the C2-1248r, D5-477, S1-954r, F-966 and SR-838 primers. Three steps were taken to verify that these amplifications were true negatives. The PCR product from rd1*/fd1* amplification of each DNA sample were purified and reamplified with each of the primer pairs fd1*/C2-1248r, rd1*/D5-477, fd1*/S1-954r, fd1*/SR-836r, and fd1*/F-966r. The PCR was modified to increase the number of amplification cycles (from 6 to 12 cycles in the early stage of amplification and from 25 to 30 cycles in the main amplification stage) to increase the sensitivity of the reaction. To rule out the possibility of inhibitors in the rumen samples preventing amplification of DNA in low abundance, the samples were cleaned with

Promega Wizard Prep and reamplified with the specific primers. None of these steps led to positive amplifications for C2-1248r, D5-477, S1-954r, F-966, or SR-836.

Because cPCR is most accurate when coamplification of target and internal control are equal, one must ensure that the concentration of internal control added to rumen DNA samples is similar to the target DNA concentration within those samples. The concentration of target DNA is unknown therefore an estimate must be obtained by amplifying rumen DNA with dilutions of internal control DNA. Rumen DNAs were co-amplified with a serial dilution of D4 internal control DNAs and a 4×10^6 dilution was found to produce best co-amplification across the samples tested. A similar test with dilutions of the *P. anaerobius* internal control showed that a 1×10^4 dilution gave best co-amplification. These dilutions of internal controls were then used with a range of concentrations of target DNAs to generate standard curves described below.

4.2.6. Standard curve construction for strain D4 and *P. anaerobius*

To enumerate strain D4 and *P. anaerobius* (and therefore by default strain D1) in rumen samples one must construct a standard curve which relates numbers of these bacterial cells to amplified DNA from cPCRs. To do this the target organism was grown in pure culture and the number of bacterial cells in the culture was determined by counting cells under a microscope. DNA was extracted from the bacterial culture and quantitated which allowed calculation of the amount of DNA extracted per cell. The extracted DNA was serially diluted and amplified with specific primers in the presence of internal control DNA which produced two PCR bands; the target and internal control DNAs. By quantifying the amount of DNA in each band and expressing these as the ratio of target/internal control one can relate the log of this ratio to the log of target DNA dilution (or log bacterial number). This generated a standard curve from which unknown target bacterial numbers was determined from known concentrations of target and internal control DNAs amplified during cPCR.

DNA was extracted from 3.7×10^9 D4 cells and was 10-fold serially diluted. Dilutions between 10^{-4} and 10^{-6} were co-amplified with a 4×10^6 dilution of the D4 internal control determined above to be in the correct range to enumerate strain D4 cells in rumen samples (Fig 4.7a) The intensities of the target and internal control DNA bands were determined and the ratio of target/internal

control plotted against log D4 bacterial cell numbers (Fig 4.7b). Similarly DNA extracted from 2.364×10^9 *P. anaerobius* cells was serially diluted and dilutions from 10^{-2} to 10^{-4} co-amplified with a 1×10^{-4} dilution of the *P. anaerobius* internal control (Fig 4.8a and b). The results show a linear response between the ratio of target/internal control intensities and D4 cell equivalents from 3.7×10^5 to 3.7×10^3 and for *P. anaerobius* cell equivalents from 2.364×10^7 to 2.364×10^5 . This means that the 4×10^6 dilution of the D4 internal control and the 1×10^{-4} dilution of the *P. anaerobius* internal control can be used for quantitation of DNA from rumen samples within these ranges for the respective bacterial populations.

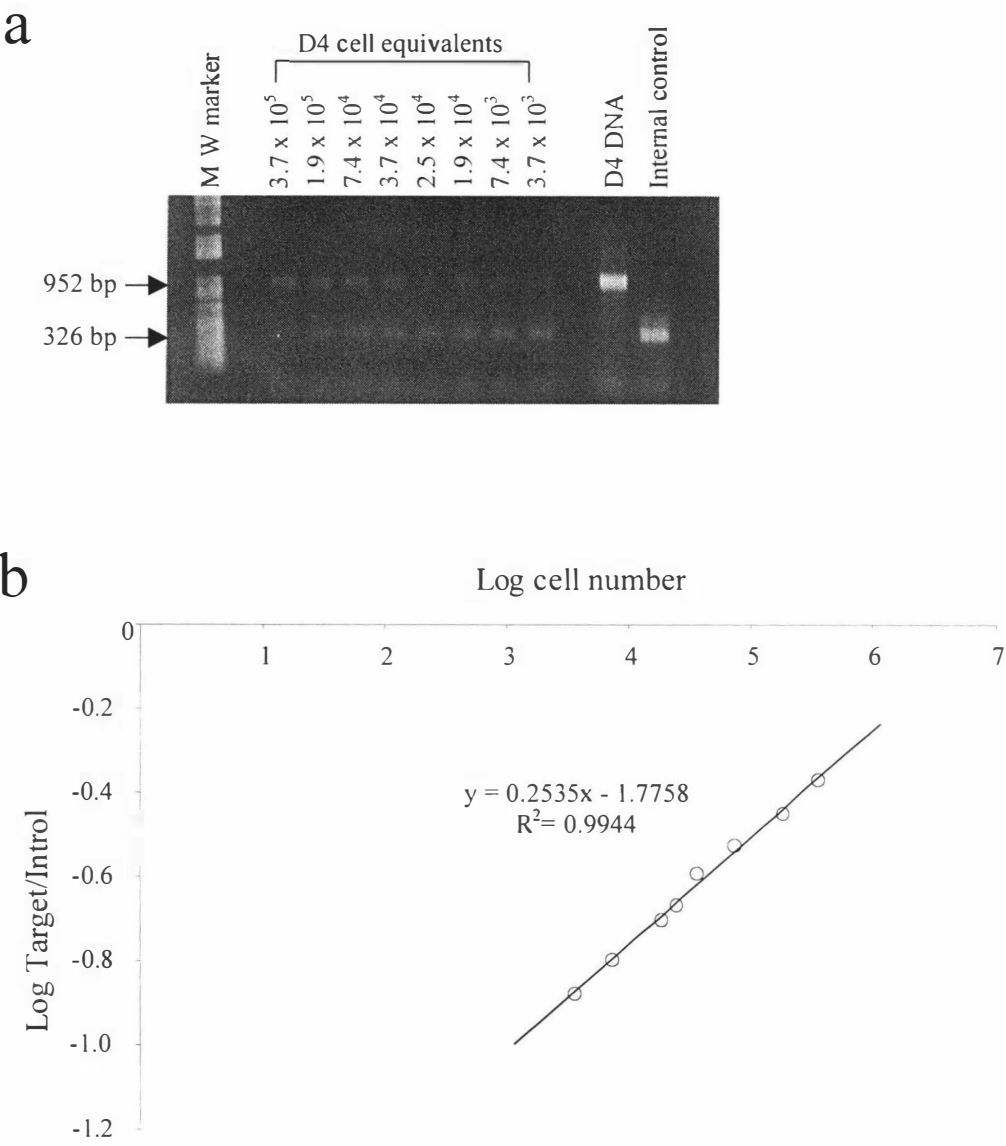


Figure 4.7. D4 standard curve

(a) DNA extracted from 3.7×10^9 D4 bacterial cells ml^{-1} was serially diluted and co-amplified with a 4×10^{-6} dilution of the D4 internal control. The results are expressed as D4 cell equivalents based on the DNA extracted per cell.

(b) The intensities of internal control and target DNAs were quantitated by staining gels in ethidium bromide, photographing the gels using Polaroid film and scanning the negative image of the photograph. The intensity of the internal control DNAs were adjusted to account for differences in binding of ethidium bromide and the ratio of target/adjusted internal control DNA intensities was plotted against the number of D4 cells on a log-log scale

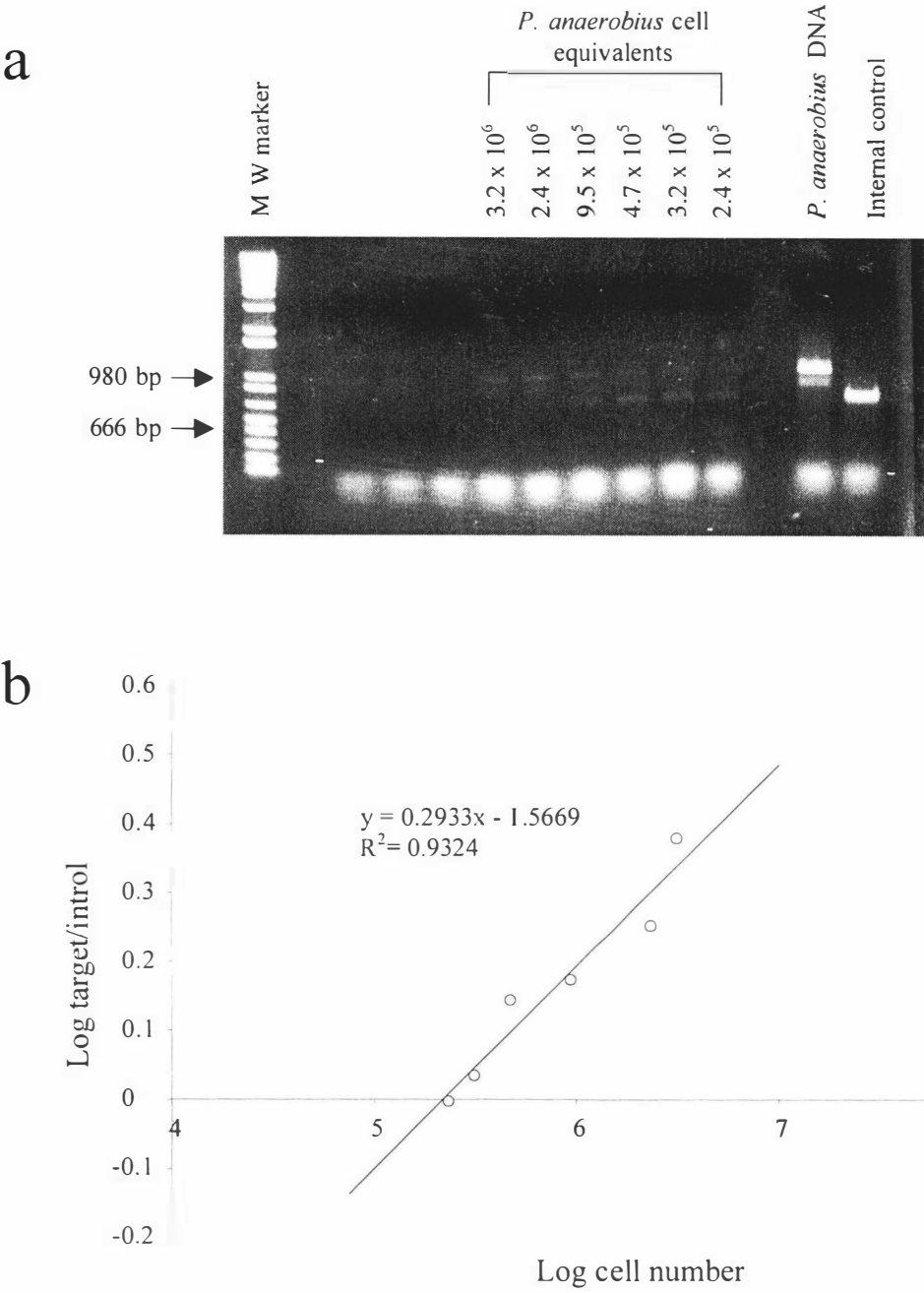


Figure 4.8. *P. anaerobius* standard curve.

(a) DNA extracted from 2.36×10^9 *P. anaerobius* cells ml⁻¹ was serially diluted and co-amplified with a 1×10^{-4} dilution of *P. anaerobius* internal control. The results are expressed as *P. anaerobius* cell equivalents based on the amount of DNA extracted per cell.

(b) The intensities of internal control and target DNA were quantitated by scanning densitometry using negative image of polaroid photographs of ethidium bromide stained gels. The ratio of the intensities of the target /adjusted internal control DNAs was plotted against the *P. anaerobius* cell number on a log scale.

4.2.7. Analysis of rumen samples

As mentioned above, rumen samples were collected from sheep before treatment started and at 24 hrs, 48hrs, 72 hrs, and 96hrs after infusion with monensin or buffer and each sample was amplified with the universal eubacterial primers fd1* and rd1* to confirm that amplifiable DNA was present. The DNA was then co-amplified using the D4-specific primer pair (D4-952r/fd1*) with a 4×10^6 dilution of the D4 internal control. Similarly, the *P. anaerobius*-specific primer pair (C980/fd1*) and a 1×10^4 dilution of the *P. anaerobius* internal control were co-amplified. A typical agarose gel showing the coamplification of target and internal control DNAs is shown in Fig 4.9. The overall results are shown in Fig. 4.10. Monensin had no effect on the population *P. anaerobius* as shown by the relatively constant population at each time point. Control animal receiving the buffer alone had similar levels of *P. anaerobius*. However in the case of strain D4, monensin had little effect over the first 48hr compared to control sheep. However, after 72hr D4 populations increased and finally reached 1.4×10^9 bacteria ml^{-1} at 96 hrs.

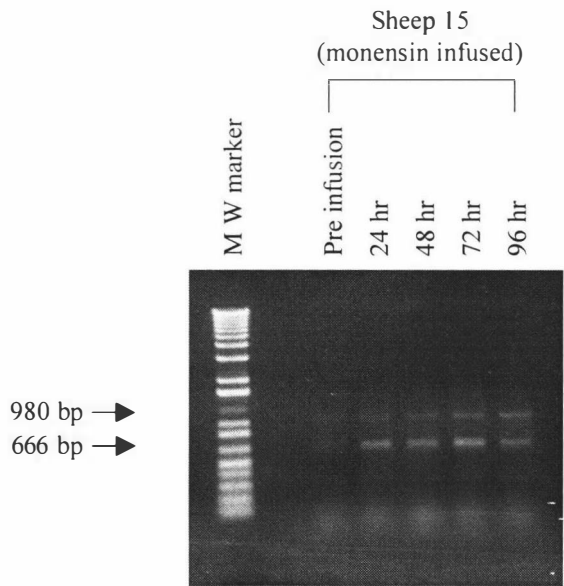


Figure 4.9. Coamplification of *P. anaerobius* internal control with DNA extracted from rumen samples.

Rumen samples were collected from sheep before treatment started and at 24 hrs, 48hrs, 72 hrs, and 96hrs after the beginning of treatment. The DNA extracted from these samples was co-amplified using the *P. anaerobius*-specific primer pair (C980/fd1*) and a 1×10^4 dilution of the *P. anaerobius* internal control. Shown above is the coamplification of DNA extracted from Sheep15 (treated with monensin).

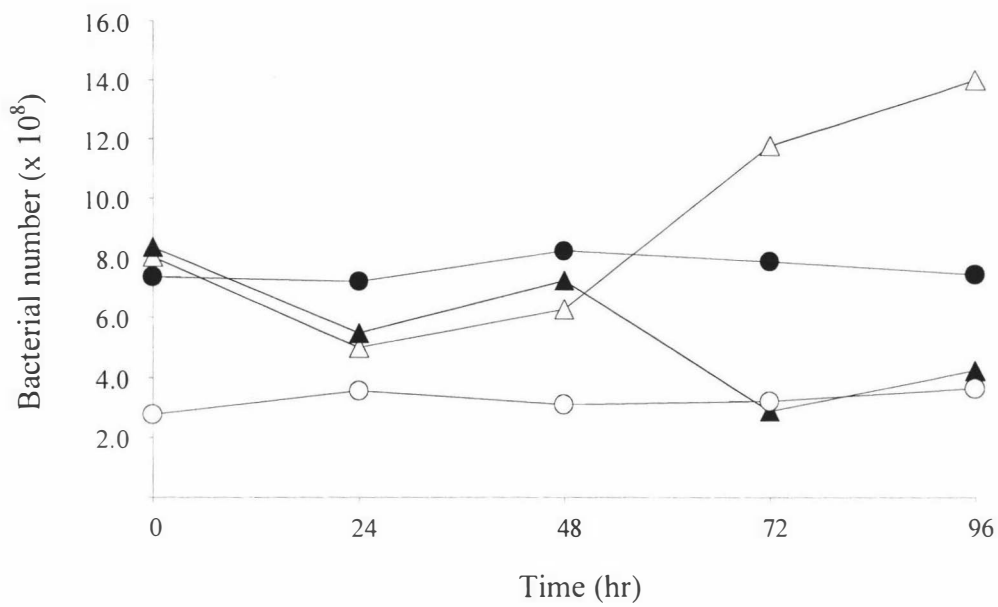


Figure 4.10. Strain D4/*F. necrophorum* and D1/*P. anaerobius* populations in the rumen of sheep infused with monensin or with buffer. DNA, extracted from rumen samples taken from sheep, was amplified with either strain D4/ *F. necrophorum* - specific primers and internal control or with D1/*P. anaerobius*-specific primers and internal control. The resulting PCR products were used to quantitate their respective populations in these samples by reference to standard curves. Sheep received infusions of monensin (open circles or triangles) or a buffer alone (filled circles or triangles) and populations of strain D4/ *F. necrophorum* (open or filled triangles) and *P. anaerobius* (open or filled circles) were measured.

Chapter 5. Discussion and Conclusions

5.1 Discussion

In this study, cPCR primers were designed for five HAP bacteria isolated from New Zealand ruminants (Attwood et al., 1998) to enable the abundance of these organisms to be estimated *in vivo*. Probes were also designed for the three previously described hyper ammonia-producing bacteria, *C. aminophilum*, *C. sticklandii* and *P. anaerobius*. The development of an enumeration technique for these organisms stemmed from the initial observations of Attwood et al., (1998) which indicated significant levels of bacteria from forage-grazed ruminants in New Zealand which were able to grow on HAP medium. They isolated fourteen colonies of distinct morphology from broth and roll tubes. There were two distinct categories of isolates: isolates C2, D1, D4, D5, and S1, which grew well on HAP medium plates and broths; and the remaining isolates which grew poorly on HAP medium plates and could not be successfully transferred to the broth form of the medium. Isolates C2, D1, D4, D5, and S1 fermented few of the commonly utilised carbon sources. D1 and D5 failed to grow on any of the carbon sources tested, while C2, D4, and S1 grew only on lactate or weakly on pyruvate. These HAP bacteria fermented amino acids and peptides to produce ammonia. Fast-growing HAP bacteria, D1, D4, produced the largest amount of ammonia in HAP liquid media and had specific activities of ammonia production of 945.5 and 748.3 nmol/min/mg, respectively. Comparatively, *C. aminophilum*, *C. sticklandii*, and *P. anaerobius* also had high specific activities of ammonia production of 676.2, 551.7 and 640.5 nmol/min/mg, respectively. (Attwood et al., 1998). Although S1 also grew rapidly in HAP liquid medium, it produced relatively small amounts of ammonia (105.3 nmol/min/mg). In this respect it resembled the rumen bacterium *Acidaminococcus fermentans* which metabolised glutamate via the hydroxy glutarate pathway to produce equimolar proportions of ammonia and acetate (Buckel and Barker, 1974). C2 and D5 produced relatively small amounts of ammonia but their specific activities of ammonia production were still higher than the common rumen bacteria. Therefore these isolates appeared to fit the description for hyper ammonia-producing bacteria such as high ammonia production, being non-proteolytic and non-saccharolytic. Although these organisms were isolated from pasture-grazed ruminants, their abundance in animals in New Zealand has not been determined, excepting for an estimation of HAP bacteria by plating rumen fluid dilutions on

HAP medium plates containing only tryptone and casamino acids as the sole nitrogen and carbon source. As pointed out by Attwood et al., (1998), many other non-HAP organisms can also grow on this medium albeit sub-optimally. Therefore cPCR probes were developed to enable us to get an estimation of the numbers of known HAP bacteria from rumen samples. Competitive PCR was chosen as the method for quantitation of these HAP bacteria as it combined the sensitivity of PCR with the specificity of the 16 S ribosomal RNA probes.

The Polymerase Chain Reaction (PCR) has been widely used for the analysis of microbial populations directly from environmental samples (Leser,1995). It has been used in the amplification of bacterial DNA before restriction enzyme analysis, improving the sensitivity in dot-blot hybridisation (Steffan and Atlas, 1988), cloning (Amann et al., 1995) and sequencing (Edwards et al., 1989; Weisburg et al., 1991; Amann et al., 1995) of ribosomal RNA genes, denaturing gradient gel electrophoresis (DGGE) and competitive PCR (Leser, 1995; Leser et al., 1995; Kobayashi et al., 1998). The main advantages of PCR are its speed and simplicity of analysis, and its specificity and sensitivity of detection of DNA from samples. In PCR, it is difficult to quantitate the amplified product due to the exponential nature of DNA amplification. PCR starts off with exponential accumulation of product, but due to many factors, accumulation of product begins to plateau. These factors include exhaustion of primers, dNTPs, inactivation of *Taq* DNA Polymerase or loss of buffering capacity in the reaction. There are inevitable variations between samples and the slightly different reaction conditions are also magnified during the amplification process. Therefore from analysis of PCR product alone it is not possible to determine the initial starting concentration of target DNA. Competitive PCR, however, uses internal DNA controls, which coamplify during the reaction and can be used to quantitate the PCR products. An internal DNA control should have the following properties:

- * it must have the same priming sites as the target DNA.
- * it must be able to be differentiated from the PCR product of the target DNA either by size in an agarose gel or else it can have a unique endonuclease restriction site within the internal control.
- * it must amplify with equal efficiency as the target DNA, or if not the same efficiency then at least amplify with a consistent difference.

In cPCR, the target and the internal control DNAs compete for primers, reagents and enzymes in the reaction. Both DNAs are subject to the same reaction conditions, and because the initial amount of target DNA is proportional to the amount of PCR product after amplification, the initial amount of target DNA can be determined using the following formula :

$$\text{Log}(N_{n1}/N_{n2}) = \text{log}(N_{O1}/N_{O2}) + n \text{ log}(\text{eff}_1/\text{eff}_2) \text{ (Zachar et al., 1993)}$$

where N_{O1}/N_{O2} is the ratio of the initial templates and N_{n1}/N_{n2} is the ratio of the products, eff_1 is the efficiency of amplification of the target DNA and eff_2 is the efficiency of amplification of the internal control. If the amplification efficiencies remain the same, the ratio of the products (N_{n1}/N_{n2}) during any cycle (n) of amplification would depend solely on the ratio of the initial templates (N_{O1}/N_{O2}). Even if $\text{eff}_1 \neq \text{eff}_2$, the quantitation is still valid if it is assumed that the ratio of $\text{eff}_1/\text{eff}_2$ is a constant value and the amplification is in the exponential phase (Zachar et al., 1993).

In a cPCR two primers are used to detect target DNA; a specific primer and an anchor primer. The specific primer determines reaction specificity while the non-specific primer, allows the reaction to proceed. Both are essential to the reaction, but only one needs to be specific to the organism of interest. The other primer can be used to "anchor" the reaction. In the case of the 16S rRNA gene the cPCRs are "anchored" at either the 5' or 3' end of the gene using the universal eubacterial primers *fdl** or *rdl** respectively. It is possible to have both PCR primers specific for the target DNA, but they both need to be balanced in terms of their lengths, G + C content and their melting temperature. The advantage of anchoring the reaction using a universal primer is that only a single specific primer has to be designed for each new organism. But it also has the disadvantage that the specific primer needs to be matched with the anchor primer in terms of the length, G + C content and the melting temperature. This makes designing of specific primers a difficult task with only a few choice of suitable primers.

In this study two primer pairs were designed for each HAP bacterial isolate but after optimisation the primer pair that performed better in regard to specificity and which amplified more efficiently was chosen for further studies. While testing for specificity against DNA from 35 ruminal bacteria, it was noticed that primers C2-

1248r, D4-952r and S1-954r performed better when their sequences were reversed and used with universal primer fd1* in a PCR. On the other hand D5-477 and D1-176 performed better when used with the universal reverse primer rd1*. The selection site of the reversed primers were closer to the universal fd1* than the universal rd1*. Also, the reversed primers were better matched with fd1* in terms of their G+C content, primer length and melting temperature and therefore performed better.

The primer selected for HAP isolate D1, D1-176, detected only DNA extracted from D1 and *P. anaerobius*. D1 and *P. anaerobius* are phylogenetically very closely related showing 99% similarity and the D1-176 probe site is present in both these organisms. One might have expected that the C-72 probe designed by Krause and Russell (1995) for *P. anaerobius* should also detect D1 DNA. But on examination of the D1 16S rRNA sequence it was found to contain a 155bp insertion from position 50 to position 204 in Helix 6 of the V1 hyper-variable region (Attwood et al., 1998). Patel et al., (1992) and Redburn and Patel, (1993) have also reported these unusual insertions in Helix 6 (nt73-82/87-97 *E. coli* numbering) of the 5' region in *Desulfotomaculum australicum* and *D. thermobenzoicum*. Spencer et al., (1984) reported similar insertions in mitochondria, while in eukaryotic small sub units, insertions in the junction between helices 7 and 11 and expand the junction to form the lower lobes of the subunit. Garrett et al., (1997) suggested that Helix 6 may play a role in ribosome assembly but the purpose of the insertions in this helix remains uncertain. The 155bp insertion in D1 16S rDNA spans the C-72 probe site described for *P. anaerobius* so that the site does not exist in the D1 16S rRNA gene. During ssrRNA phylogenetic analysis such variable regions are excluded from analysis. This analysis would therefore still show that D1 and *P. anaerobius* are very similar. This also explains the reason for the C-72 oligonucleotide probe not detecting D1 DNA when it was used by Attwood et al., (1998). This study had investigated the presence of HAP bacterial DNA from rumen samples of deer, sheep and dairy cow, using rDNA probes and dot blot probing. To detect both *P. anaerobius* and D1 in the present study, another primer, C-980 was designed. The C-980 probe amplified well with DNA from both bacteria while not amplifying with DNA from any of the 35 organisms tested.

Specificity tests with probes for isolates C2 and D5 showed that they were specific for their respective target organisms and did not amplify any of the other 35 DNA samples tested. However probes for S1 and D4 amplified with their own DNA and with DNA from *P. asaccharolyticus* and *F. necrophorum* respectively. The cross reaction of the S1 probe with *P. asaccharolyticus* DNA was unexpected as their 16S rDNA sequences showed significant differences (96.9% similarity) and the probe site had 6 differences out of 20bp. However, the 9 bases at the 3' end of the S1 probe exactly match the *P. asaccharolyticus* sequence. It is known that the 3' region of primers is the most important area for primer annealing during PCR (Giovannoni, 1991) and this has probably allowed probe hybridization and elongation to occur during the reaction. The cross reaction of the D4 probe with *F. necrophorum* may have been predicted as these organisms are closely related (98.8% similarity, Attwood et al., 1998). Inspection of the probe site in *F. necrophorum* shows that there are 5 differences from the D4 sequence. These differences are distributed throughout the probe site.

The accuracy of cPCR depends on equal coamplification of target and internal control DNAs. (Raeymakers, 1993) although DNAs with different efficiencies of amplification can be quantitated if the difference in efficiency remains the same. Reilly and Attwood (1998) investigated the efficiency of amplification of a target DNA from *Clostridium proteoclasticum* (830 bp) with its corresponding internal control (480 bp). These DNAs differed in size by 350 bp but amplified with essentially the same efficiency. In the present study internal controls for the 7 primers were developed using the technique of identifying unique restriction endonuclease sites within the target 16S rDNA gene sequence and using appropriate restriction endonuclease to cut out the intervening DNA. This allowed the terminal fragments to be religated forming a deleted DNA fragment which was smaller but which retained the primer sites at each end. Appropriate restriction sites (ie at a convenient distance from the primer site) were not always available, and often it required obscure restriction enzymes to achieve an appropriate deletion. As an internal control had to be made for each primer set, it was a time consuming process and it contributed to a major portion of the time taken to complete this study.

Once constructed, the internal controls were coamplified with their respective target DNAs. In the case of D4 and D1/*P. anaerobius* that were identified from rumen samples in this study it was found that a single concentration of their respective

internal controls was sufficient to coamplify target DNA over a range of about 10^3 to 10^4 cells. However if the range of bacterial number was greater than this then different dilutions of the internal control would have to be used to accommodate this range. The detection limits for D4 and *P. anaerobius* DNA were determined by amplifying serial dilutions of the DNA extracted from a known number of D4 and *P. anaerobius* cells with their specific primers. The highest dilution point at which the target DNA and the specific primer amplified was determined. This was found to be a 1×10^5 dilution for D4 (equivalent to 3.7×10^3 cells per reaction). This is comparable to the detection limit determined for the proteolytic organisms *C. proteoclasticum*, which was detectable at 2.5×10^3 cells per reaction (Reilly and Attwood, 1998). However *P. anaerobius* DNA was detectable only down to a 1×10^3 dilution (equivalent to 2.364×10^6 cells per reaction) indicating a much less sensitive assay for this organism. The lower sensitivity of the D1/*P. anaerobius* probe could be due to less efficient primer binding, or alternatively some physical constraint on the accessibility of the C-980 primer to the target site. The stable stem-loop secondary structures of rRNA are known to influence oligonucleotide binding during in situ probing (Amann et al., 1995) and it may be possible that stable secondary structures in rDNA may also affect primer binding.

The sensitivity of the cPCR assay was compared with that of the 16S rRNA dot blot hybridisation work done by Krause and Russell (1996). Krause and Russell, (1996) expressed 16S rRNA hybridised as a percentage of the total rRNA in the sample. In their continuous culture studies with predominant rumen bacteria they found that *C. aminophilum*, *C. sticklandii*, and *P. anaerobius* comprised approximately 1% of the total rumen 16S rRNA. The radiolabelled probes used were able to detect down to 0.2 % of the total 16S rRNA present. If one assumes a rumen bacterial population of 1×10^{10} cells per ml then this equates to $2-3 \times 10^7$ cells per ml of rumen fluid. Even though the radioactive probes enabled them to follow individual bacterial species in defined co-cultures, the technique is unlikely to be sensitive enough to detect individual strains in a complex ecosystem such as the rumen, where larger more diverse microbial populations reside

A disadvantage of the cPCR technique is that it does not discriminate between dead and living bacterial cells. Therefore amplification of DNA from dead cells is likely to overestimate viable bacterial numbers. Oligonucleotide probes targeting rRNA

more accurately reflect the metabolic status of a microbial population. As the rRNA content in the cell changes during the growth of a cell, oligonucleotide probing provides an approximate value for the relative cell numbers. This in turn reflects their contribution to total metabolic activity (Stahl et al., 1988). However, estimation of total rRNA depends on the use of universal probes. Some work on universal probes has shown variation in dissociation temperatures which could bias quantification of microbial populations from environmental samples (Zheng et al., 1996). The most appropriate approach to get an accurate assessment of the microbial population in an environment sample would therefore be to determine absolute cell numbers by cPCR and estimate rRNA as a percentage of the total rRNA by oligonucleotide probing (Reilly and Attwood, 1998).

Having designed and tested probes for HAP bacteria they were then applied to the quantitation of these organisms in rumen samples collected from an animal trial. The animal trial was designed to assess the effect of monensin on rumen HAP bacteria and was carried out with 8 fistulated Romney wether sheep continuously fed a diet of chaffed lucerne. Monensin was administered via intra-ruminal infusion to 4 animals so that each animal received the recommended dose per day (23mg). The average rumen volume of the monensin treated sheep was 3 litres and assuming a rumen turnover every 8 hrs, then the concentration of monensin within the rumen would have been approximately 3.7 μ M. Competitive PCR was able to detect D1/*P. anaerobius* and D4/*F. necrophorum* in the rumen samples, but failed to detect C2, D5, S1, *C. aminophilum* or *C. sticklandii*. The populations of D1/*P. anaerobius* seen in the experimental sheep ranged from 2.78 to 7.86 $\times 10^8$ cells/ ml while D4/ *F. necrophorum* populations varied from 2.88 $\times 10^8$ to 1.4 $\times 10^9$ cell/ml. These numbers are on the threshold of dot blot detection when probing ribosomal RNA and DNA. Therefore using the dotblot hybridization technique of Krause and Russell (1996) the populations of HAP bacteria observed in this study may have been missed out due to its lower sensitivity. Attwood et al., (1998) used the C-72 primer, designed by Krause and Russell (1996) for detection of *P. anaerobius* using a dot blot format for detection of bacterial DNAs extracted from rumen samples of deer, cow and sheep. As discussed above the C-72 probe would not have detected D1 but it is also likely that the sensitivity of the technique would not have allowed detection of *P. anaerobius* either.

The genus *Peptostreptococcus* is composed of non-spore forming gram positive, anaerobic cocci. They form part of the normal flora of man and animals. The bacteria metabolise amino acids and peptones to acetic, butyric, *iso*-butyric, *iso*-valeric, or *iso*-caproic acids (Holdeman et al., 1984).

P. anaerobius is found in the natural cavities of man and animals. It produces hydrogen sulphide in Sulphide-Indole motility medium and breaks down peptone to release ammonia. It ferments glucose and maltose weakly but does not ferment lactose. This species is often a part of the flora of the gingiva of human suffering from gingivitis or periodontal diseases. Russell et al.(1988), studied *P. anaerobius* isolated from rumen samples in detail and found that it was unable to utilise any of the carbohydrates tested as sole energy source. It grew rapidly under anaerobic conditions with trypticase (15g/liter) as the only energy source and the products after 24hrs were NH₃, acetate, *iso*-butyrate, *iso*-valerate, methyl butyrate, valerate and *iso*-caproate. H₂ was also detected. Ammonia was not produced if either glutamate or glycine were provided as a single amino acid. Gelatin was not hydrolysed, casein was not digested and indole was not produced. *P. anaerobius* produced large amounts of H₂S in media containing trypticase and cysteine. It could not use pyruvate, lactate or mannitol or reduce sulphate to H₂S. It did not produce ammonia from urea. The cells did not produce spores, were non-motile and stained Gram positive. It could not grow in the presence of 5 µM monensin. In comparison D1 is also anaerobic and grows rapidly on HAP medium and broths, but does not grow on any carbon substrates. It utilises nitrogen sources well and produces great amounts of ammonia. It does not grow in 5µM monensin. Fermentation products were acetate, *iso*-butyrate, *iso*-valerate and *iso*-caproate. It also produces H₂S. Therefore from the above comparison it could be concluded that *P. anaerobius* and D1 are very similar phenotypically. They showed many similar metabolic activities which might have enabled them to utilise peptides and amino acids for growth and multiplication and thrive better in the rumen environment when compared with *C. aminophilum* and *C. sticklandii*.

Monensin did not show any inhibitory effect on the D1/*P.anaerobius* population in this study. These bacteria maintained steady levels throughout the study period. These results when compared with those from the in vitro monensin study of Krause and Russell (1996) revealed an interesting phenomenon. They studied the effect of monensin on HAP bacteria grown together with a 10⁸ dilution of

predominant ruminal bacteria in continuous cultures. The HAP bacteria, *C. aminophilum*, *C. sticklandii*, and *P. anaerobius* accounted for less than 5% of the total 16S rRNA in the continuous culture before monensin supplementation. At 0.1 μ M monensin concentration *P. anaerobius* and *C. aminophilum* accounted for 1.3 to 1.5% of the total 16S rRNA, while *C. sticklandii* accounted for less than 0.7%. When the monensin levels were raised to 0.3 μ M, *C. sticklandii* could not be detected, and at 0.6 μ M monensin concentration *P. anaerobius* slipped below detectable levels. However *C. aminophilum* continued to grow and increased in population even when the monensin concentration reached 5 μ M. It was demonstrated that *C. aminophilum* was not monensin resistant in batch cultures containing 5 μ M monensin but showed resistance only in low-dilution rate continuous cultures which mimicked the rumen. It seems that *C. aminophilum* may have adapted under continued exposure to low dilutions of monensin and hence show resistance. Thus the *C. aminophilum* monensin resistance shown was a growth rate dependent phenomenon. Therefore, in continuous culture at least, *P. anaerobius* is susceptible to a monensin concentration of 0.6 μ M whereas the results of the present study show they were unaffected by an *in vivo* monensin concentration of 3.7 μ M. However, it is possible that monensin could bind to monensin-resistant bacteria and feed particles, thereby making its effective concentration in the rumen much lower than that calculated from infusion rate, ruminal volume and turnover.

The *in vivo* D4/*F. necrophorum* population detected in this study ranged from 3×10^8 to 1.4×10^9 bacteria /ml. In the initial stages following monensin administration there was a drop in D4/*F. necrophorum* numbers which was paralleled in control animals. However after 48hr there was an increase in population which continued until the end of the study period. Attwood et al., (1998) reported that D4 showed some resistance to monensin, with weak growth at 5 μ M monensin.

Elimination of monensin-sensitive HAP bacteria and other rumen bacteria may have relieved D4/*F. necrophorum* from inhibitory interactions from these competitors. This presumably would enable improved access to peptides and amino acids for growth and therefore survival in the rumen. The growth habits of D4/*F. necrophorum* also indicate that they may be adapted to growth in the rumen environment. *F. necrophorum* is an obligately anaerobic Gram negative rod. It metabolises peptone producing butyrate, propionate, acetate, sometimes producing

lactate and succinate. It converts lactate to propionate and releases large amount of gas in peptone-yeast extract glucose agar deeps. It was isolated from the natural cavities of man and animals, necrotic lesions, abscesses of man, liver abscesses and from foot rot of cattle. These are protein rich environments where peptides and amino acids are likely to be available in good supply. D4 by comparison is a Gram-negative rod and it grows well on HAP medium producing large amounts of ammonia. It produces acetate, *n*-butyrate and a small amount of propionate. It grows on lactate, weakly on pyruvate, but no carbohydrates were fermented. It weakly produced H₂S. D4 is fast growing and prefers tryptone (peptides) as its nitrogen source. From the above comparisons it is evident that D4/*F. necrophorum* is suited to occupy the peptide/amino acid fermenting niche in the rumen.

The increase of D4/*F. necrophorum* with monensin infusion may be showing a similar trend to *C. aminophilum* which showed resistance to monensin in continuous cultures containing low concentration of monensin (Krause and Russell, 1996). Krause and Russell, 1996 demonstrated that *C. aminophilum* was monensin resistant only in low-dilution rate continuous cultures which mimicked the rumen. As monensin resistance was not seen in batch cultures it seems that *C. aminophilum* may have adapted under continued exposure to low dilutions of monensin and hence show resistance. It is possible that D4/*F. necrophorum* has responded in a similar manner to monensin infusion and expanded its population to occupy the peptide and amino acid-fermenting niche vacated by monensin-sensitive HAP bacteria.

The presence of D1/*P. anaerobius* in the rumen at an average population of around 5×10^8 and D4/*F. necrophorum* at 7×10^8 cells per ml rumen fluid, suggests that they may form from 5 to 7% of the total population. Numerically this is quite low but given that D1 and D4 both have high specific activities of ammonia production (945.5 and 748 nmoles/min/mg respectively), their contribution to ammonia production in the rumen could be significant.

The present study showed that the cPCR technique was not able to detect D5, S1, *C. aminophilum* or *C. sticklandii* from rumen samples as these bacterial numbers were either below the detectable limit of cPCR technique or not present at all. The failure of conventional PCR to detect any amplification of 16S rDNA of these

organisms suggests that if they are present then they are at very low levels. Analysis of rRNA sequence showed that C2 was most closely related to *Eubacterium* sp. strain SC87K (94.4%) and *Eubacterium nodatum*. The genus *Eubacterium* includes a diverse assemblage of bacterial species in 3 sub groups: those producing butyric acid usually in combination with other volatile fatty acids; those producing combinations of lactate, acetate and formate with H₂ gas, and those producing little if any detectable fermentation acids. The genus also includes some species that are phylogenetically distant from each other. *Eubacterium* species are found in the oral cavity of man and animals. Several species are found in the rumen including *E. ruminantium*, *E. limosum* and *E. cellulosolvens* (Moore and Holdeman-Moore, 1984) while proteolytic isolates resembling *E. budayi* were found in pasture-grazed ruminants in New Zealand (Attwood and Reilly, 1995). All these species are able to ferment carbohydrates and produce only small quantities of ammonia in peptone-containing media (Moore and Holdeman-Moore, 1984). In comparison, C2 did not grow on any commonly utilised carbohydrate sources except weakly on pyruvate. Growth rate and nitrogen preference studies indicate that it grew poorly on media containing gelatin or casein, weakly on tryptone or casamino acids and had significant growth only on HAP medium, which supplied both amino acids and peptides. This indicated that C2 required high levels of both peptides and amino acids to achieve significant growth. The 16S rRNA sequence of D5 was most closely related to *Ruminococcus flavefaciens*. *R. flavefaciens* is an important rumen bacterium being involved in cellulose and hemicellulose degradation and pectin hydrolysis. However they have very little ability to ferment amino acids or peptides and require ammonia as their main source of nitrogen. In comparison, D5 grew poorly on HAP medium plates and in broths. It did not ferment any of the commonly tested carbon sources, and produced only small amounts of ammonia. It is most likely that C2 and D5 were not detected in significant populations because they are slow growing, have limited ability to use substrates other than peptides or amino acids, and they need to have high levels of peptides/amino acids to attain significant growth. Therefore they may be present in smaller population (less than $\sim 2.5 \times 10^3$ cells/ml) in the rumen.

The failure to detect S1 from rumen samples on the other hand seems to be strange. The 16S rDNA sequence of S1 was most closely related to that of *P. asaccharolyticus* (96.9%). *P. asaccharolyticus* is a Gram-positive coccus which

does not ferment any common carbohydrate sources but metabolises pyruvate to acetate and butyrate. Lactate is not utilised but purines and pyrimidines are metabolised to acetic and lactic acids, CO₂ and ammonia. S1 grew well on HAP medium and broths, grew weakly on pyruvate and lactate but did not ferment any of the other commonly used carbon sources. It produced little ammonia although it grew well on HAP medium and broths. The fermentation products were acetate, *n*-butyrate and very small amounts of propionate. It showed weak signs for H₂S production and was also sensitive to 5µM monensin. Compared to D1 and D4, S1 had a similar rate of growth on HAP medium, was at least as metabolically versatile as D4 and S1 and was able to grow on lactate whereas D1 could not. S1 produced much less ammonia when grown on HAP medium suggesting that it has a different method of peptide and amino acid metabolism. S1 may follow a similar pathway as the rumen bacterium *Acidaminococcus fermentans* which metabolises glutamate via the hydroxy glutarate pathway, producing ammonia and acetate in equimolar proportions (Buckel and Barker, 1974). Factors other than growth rate and metabolic ability may be determining the S1 population *in vivo*. If the phenotypic characteristics of S1 are examined, they are more similar to the genus *Acidaminococcus*, although the phylogenetic analysis places it closest to *P. asaccharolyticus*. As *P. anaerobius* and D1 were detected by cPCR it seems safe to assume that cPCR would also detect *P. asaccharolyticus* and S1 if present at similar levels. It appears that D1/*P. anaerobius*, and D4/*F. necrophorum* are better adapted to grow on the experimental diet than S1/*P. asaccharolyticus* and therefore are able to establish higher populations.

5.2 Conclusions

The cPCR probes developed in this study have enabled *in vivo* detection and quantitation of HAP bacteria. Detection and enumeration of HAP bacteria by conventional culture methods has been hampered in the past by the absence of a completely selective medium. This has been overcome to an extent by designing specific probes for each of these bacteria and using these probes in cPCRs to enumerate individual HAP bacterial population in a given rumen sample. However, the cPCR is a time-consuming technique, as it requires the construction of an internal control and a standard curve for each target bacterium. The study showed that cPCR has the sensitivity of PCR and the specificity of probes designed to anneal with target regions of 16S rDNA genes. Also by including internal control

DNAs and making standard curves the technique is able to give quantitative information which is expressed as a bacteria number rather than as a percentage of the total population. These features are well suited for molecular investigation of microbial populations in a densely populated and complex ecosystem like the rumen.

The study showed that D1/*P. anaerobius* and D4/*F. necrophorum* were present at significant levels in rumen samples collected from an animal trial investigating the effect of monensin infusion on HAP bacterial populations. Monensin did not affect the level of D1/*P. anaerobius* while D4/*F. necrophorum* populations increased nearly two-fold due to monensin infusion. Therefore monensin can not be credited as having an inhibitory action on rumen HAP bacteria *in vivo*, at least on the HAP bacteria that were detected in this study. Other, previously described, HAP bacteria could not be detected at all in the rumen samples suggesting that they either are not present or present at very low levels. It is possible that the experimental diet of chaffed lucerne may not have provided peptides and amino acids in sufficient concentrations for some of these HAP bacteria to establish detectable populations.

Future research should be focused on the use of cPCR for quantitation of the HAP bacterial population from the rumen samples of fresh forage grazed ruminants. Such experiments conducted on ruminant species such as sheep, cattle and deer on pasture may give a better indication of true HAP bacterial populations under New Zealand's production conditions. Further research is also required to determine the contribution of individual HAP bacteria to ammonia production in the rumen so that wastage of nitrogen as ammonia from high nitrogen diets can be estimated.

Chapter 6. References cited

- Abou Akkada, A. R., and Blackburn, T. H.** (1963) Some observations on the nitrogen metabolism of proteolytic bacteria. *J. Gen. Microbiol.* **31**: 461-469.
- Allison, M. J.** (1970) Nitrogen metabolism of ruminal microorganisms, pp 456-472. In Phillipson, A.T., (ed.), Physiology of digestion and metabolism in the ruminant. Oriel Press, Newcastle-upon-Tyne, United Kingdom.
- Amann, R. I., Ludwig, W and K.-H. Schleifer, K. H.** (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev.* **59**:143-169.
- Armstead, I. P. and Ling, J. R.** (1993) Variations in the uptake and metabolism of peptides and amino acids by mixed ruminal bacteria in vitro. *Appl. Environ. Microbiol.* **59**:3360-3366.
- Attwood, G. T., Klieve, A. V., Ouwerkerk, D. and Patel, B. K. C.** (1998) Ammonia-hyperproducing bacteria from New Zealand ruminants. *Appl. Environ. Microbiol.* **64**:1796-1804.
- Attwood, G. T. and Reilly, K.** (1995) Identification of proteolytic rumen bacteria isolated from New Zealand cattle. *J. Appl. Bacteriol.* **79**:22-29
- Avgustin, G., Wallace, R. J. and Flint, H. J.** (1997) Phenotypic diversity among rumen isolates of *Prevotella ruminicola*: proposal of *Prevotella brevis* sp. nov., *Prevotella bryantii* sp. nov., and *Prevotella albensis* sp. nov., and a redefinition of *Prevotella ruminicola*. *Int. J. Syst. Bacteriol.* **47**:284-288
- Barker, H. A.** (1981). Amino acid degradation by anaerobic bacteria. *Ann. Rev. Biochem.* **50**:23-40.
- Bej, A. K., Mahubani, M. H. and Atlas, R. M.** (1991) Amplification of nucleic acids by Polymerase Chain Reaction (PCR) and other methods and their applications. *Crit. Rev. Biochem. Mol. Biol.* **26**:301-334.
- Bej, A. K. and Mahubani, M. H.** (1992) Application of the Polymerase Chain Reaction in environmental microbiology. *PCR Meth. Applicat.* **1**: 151-159.
- Blackburn, T. H.** (1965) Nitrogen metabolism in the rumen, pp.322-334. In Dougherty, R. W., Allens, R. S., Burroughs, W., Jacobson, N. L and McGilliard, A. D., (ed.), Physiology of Digestion in the Ruminant. Oriel Press, Newcastle-upon-Tyne, United Kingdom.
- Blackburn, T. H. and Hobson, P. N.** (1960) Proteolysis in the sheep rumen by whole and fractionated rumen contents. *J. Gen. Microbiol.* **22**:272-281.

Bladen, H. A., Bryant, M. P., and Doetsch, R. N. (1961) A study of bacterial species from the rumen which produce ammonia from protein hydrolyzate. *Appl. Microbiol.* **9**:175-180.

Briesacher, S. L., May, T., Grigsby, K. N., Kerley, M. N., Anthony, R. V. and Paterson, J. A. (1992) Use of DNA probes to monitor nutritional effects on ruminal prokaryotes and *Fibrobacter succinogenes* S85. *J. Anim. Sci.* **70**:289-295.

Brock, F. M., Forsberg, C. W., and Buchanan-Smith, J. G. (1982) Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. *Appl. Environ. Microbiol.* **44**:561-569.

Broderick, G. A., Wallace, R. J. (1988) Effects of dietary nitrogen source on concentrations of ammonia, free amino acids and fluoroscamine-reactive peptides in the sheep rumen. *J. Anim. Sci.* **66**:2233-2238.

Bryant, M. P. (1956) The characteristics of strains of *Selenomonas* isolated from bovine rumen contents. *J. Bacteriol.* **72**:162-167.

Bryant, M. P., Small, N., Bouma, C., and Chu, H. (1958b) *Bacteroides ruminicola* sp. nov. and *Succinimonas amylolytica* the new genus and species. *J. Bacteriol.* **76**:15-23.

Buckel, W., and Barker, H. A. (1974) Two pathways of glutamate fermentation by anaerobic bacteria. *J. Bacteriol.* **117**:1248-1260.

Chalupa, W. (1976) Degradation of amino acids by the mixed rumen microbial population. *J. Anim. Sci.* **43**:828-834.

Chen, G., and Russell, J. B. (1988) Fermentation of peptides and amino acids by a monensin-sensitive ruminal peptostreptococcus. *Appl. Environ. Microbiol.* **54**:2742-2749.

Chen, G., and Russell, J. B. (1989a) More monensin-sensitive, ammonia-producing bacteria from the rumen. *Appl. Environ. Microbiol.* **55**:1052-1057.

Chen, G., and Russell, J. B. (1989b) Sodium-dependent transport of branch-chain amino acids by a monensin-sensitive ruminal *Peptostreptococcus*. *Appl. Environ. Microbiol.* **55**:2658-266.

Chen, G. J., and Russell, J. B. (1990) Transport and deamination of amino acids by a gram-positive, monensin-sensitive ruminal bacterium. *Appl. Environ. Microbiol.* **56**:2186-2192.

Chen, G.J., and Russell, J. B. (1991) Effect of monensin and a protonophore on protein degradation, peptide accumulation and deamination by mixed ruminal microorganisms *in vitro*. *J. Anim. Sci.* **69**:2196-2203.

Chen, G. J., Strobel, H. J., Russell, J. B. and Sniffen, C. J. (1987) Effect of hydrophobicity on utilization of peptides by ruminal bacteria *in vitro*. *Appl. Environ. Microbiol.* **53**:2021-2025.

Chen, M., and Wolin, M. J. (1979) Effect of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccharolytic bacteria. *Appl. Environ. Microbiol.* **38**:72-77.

Chow, J. M., and Russell, J. B. (1994) Binding of radiolabelled monensin and lasalocid to ruminal microorganisms and feed. *J. Anim. Sci.* **72**:1630-1635.

Cooper, P. B. and Ling, J. R. (1985) The uptake of peptides and amino acids by rumen bacteria. *Proc. Nutrl. Soc.* **44**:144A (abs).

Dinius, D. A., Simpson, M. E. and Marsh, P. B. (1976) Effect of monensin fed with forage on digestion and the ruminal ecosystem of steers. *J. Anim. Sci.* **42**:229-234.

Edwards, U., Rogall, T., Blocker, H., Emde, M. And Bottger, E. C. (1989) Isolation and direct complete nucleotide determination of entire genes. Characterisation of a gene encoding 16S ribosomal RNA. *Nucleic Acids Res.* **17**:7843-7853.

Egan, R., and Ulyatt, M. J. (1980) Quantitative digestion of fresh herbage by sheep. VI. Utilisation of nitrogen in five herbages. *J. Agric. Sci. Camb.* **94**:47-56.

El-Shazly, K. (1952a) Degradation of protein in the rumen of the sheep. I. Some volatile fatty acids including branched-chain isomers found *in vivo*. *Biochem. J.* **51**:640-646.

El-Shazly, K. (1952b) Degradation of protein in the rumen of the sheep. II. The action of the rumen microorganisms on amino acids. *Biochem. J.* **51**: 647-653.

Eschenlauer, S. (1994) Les bacteries fermentrices obligatoires des peptides et acides amines du rumen. Universite d'Auvergne, Clermont-Ferrand, France.

Fulghum, R. S., and Moore, W. E. C (1963) Isolation, enumeration, and characteristic of proteolytic ruminal bacteria. *J. Bacteriol.* **85**: 808-815.

Fox, G. E., Stackebrandt, E., Hespell, R. B., Gibson, J., Maniloff, J., Dyer, T. A., Wolfe, R. S., Balch, W. E., Tanner, R. S., Magrum, L. J., Zablem, L. B., Blackmore, R., Gupta, R., Bonen, L., Lewis, B. J., Stahl, D. A., Luehrsen, K. R., Chen, K. N. and Woese, C. R. (1980) The phylogeny of prokaryotes. *Science* **209**:457-463.

Garrett, R. A., Ungewickell, E., Newberry, V., Hunter, J., and Wagner, R. (1977) An RNA core in the 30S ribosomal subunit of *Escherichia coli* and its structural and functional significance. *Cell Biol. Int. Rep.* 1:487-502

Giovannoni, S. (1991) The polymerase chain reaction, pp176-203 *In* E. Stackebrandt and M. Goodfellow (ed.) *Nucleic Acid Techniques in Bacterial Systematics*, John Wiley and Sons Ltd., Chichester, England.

Hespell, R. B. (1984) Influence of ammonia assimilation pathways and survival strategy on ruminal microbial growth. pp.346-356. *In* F. M. C. Gilchrist and R. J. Mackie (ed.) *Herbivore Nutrition in the Subtropics and Tropics*, Science Press, South Africa.

Hino, T. and Russell, J. B. (1985) The effect of reducing equivalent disposal and NADH/NAD on the deamination of amino acids by intact and cell-free extracts of rumen microorganisms. *Appl. Environ. Microbiol.* 50:1368-1374.

Holdeman, L. V., Kelley, R. W., and Moore, W. E. C. (1984) Genus *Peptostreptococcus*, pp. 1082-1092. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's Manual of Systematic Bacteriology*, vol. 2. Williams & Wilkins Co., Baltimore, MD, USA.

Holdeman, L. V., Kelley, R. W., and Moore, W. E. C. (1984) Genus *Fusobacterium*, p.633-636. *In* N. R. Kreig and J. G. Holt (ed.), *Bergey's Manual of Systematic Bacteriology*, vol. 1. Williams and Wilkins Co., Baltimore, MD, USA.

Hungate, R. E. (1957) Microorganisms in the rumen of cattle fed constant ration. *Can. J. Microbiol.* 3:289-311.

Hungate, R. E. (1966) *The Rumen and its Microbes*. Academic Press, New York.

Innis, M. A., and Gelfand, D. H. (1990) Guidelines for designing PCR primers and optimization of PCRs. pp 3-12. *In* Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (ed.) *PCR Protocols: A Guide to Methods and Applications*, Academic Press, San Diego, CA, USA.

Johns, A. T. (1955) Pasture quality and ruminant digestion. 1. Seasonal change in botanical and chemical composition of pasture. *NZ. J. Sci. Technol.* A37: 301-311.

Kobayashi, Y., Koike, S., Nagamoto, Y., Forster, R. J. and Teather, R. M. (1998) Development of competitive PCR assays for 4 different cellulolytic and hemicellulolytic bacteria from the rumen. pp.123 *In* Abstracts of the M.I.E Bioforum.

Kopečný, J. and Wallace, R. J. (1982) Cellular location and some properties of proteolytic enzymes of some rumen bacteria. *Appl. Environ. Microbiol.* 43:1026-1033.

- Krause, D. O., and Russell, J. B.** (1996) An rRNA approach for assessing the role of obligate amino acid-fermenting bacteria in ruminal amino acid deamination. *Appl. Environ. Microbiol.* **62**:815-821.
- Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. and Pace, N. R** (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proc. Natl. Acad. Sci. USA.* **82**:6955-6959.
- Larsen, N., Olsen, G. J., Maidak, B. L., McCaughey, M. J., Overbeck, R., Macke, T. J., Marsh, T. L. and Woese, C. R.** (1993) The Ribosomal Database Project. *Nucleic Acids Res.* **21**:3021-3023.
- Lewis, D.** (1955) Amino acid metabolism in the rumen of sheep. *Brit. J. Nutr.* **9**:215-230.
- Lewis, D. and Elsdon, S. R.** (1955) The fermentation of L-threonine, L-serine, L-cysteine and acrylic acid by a gram negative coccus. *Biochem. J.* **60**:683-692.
- Lee, S. Y., Bollinger, J., Bezdicek, D. and Ogram, A.** (1996) Estimation of the abundance of an uncultured soil bacterial strain by a competitive PCR method. *Appl. Environ. Microbiol.* **62**:3787-3793.
- Leedle, J. A. and Hespell, R. B.** (1980) Differential carbohydrate media and anaerobic replica plating techniques in delineating carbohydrate-utilizing subgroups in rumen bacterial populations. *Appl. Environ. Microbiol.* **39**:709-719
- Leser, T. D.** (1995) Quantitation of *Pseudomonas* sp. strain B13 (FRI) in the marine environment by competitive polymerase chain reaction. *J. Microbiol. Methods* **22**:249-269.
- Leser, T. D., Boye, M. and Hendriksen, N. B.** (1995) Survival and activity of *Pseudomonas* sp. strain B13 (FRI) in a marine microcosm determined by quantitative PCR and an rRNA-targeting probe and its effect on the indigenous bacterioplankton. *Appl. Environ. Microbiol.* **61**:1201-1207.
- Ling J. R. and Armstead, I. P.** (1995) The *in vitro* uptake and metabolism of peptides and amino acids by five species of rumen bacteria. *J. Appl. Bacteriol.* **78**: 116-124.
- McKain, N., Wallace, R. J. and Watt, N. D.** (1992) Selective isolation of bacteria with dipeptidyl aminopeptidase type I activity from the sheep rumen. *FEMS Microbiol. Lett.* **95**:169-174.
- McSweeney, C. S., Mackie, R. J., Odenyo, A. A. and Stahl, D. A.** (1993) Development of an oligonucleotide probe targeting 16S rRNA and its application for detection and quantitation of the ruminal bacterium *Synergistes jonesii* in a mixed population chemostat. *Appl. Environ. Microbiol.* **59**:1607-1612.

- May, T., Kerley, M. S. and Williams, J. E.** (1993) Supplemental protein influences on carbohydrate degradation and bacterial 16S ribosomal ribonucleic acid. *J. Dairy. Sci.* **76**:3479-3489.
- Mullis, K. B. and Faloona, F. A.** (1987) Specific synthesis of DNA *in vitro* via a polymerase catalyzed chain reaction. *Meth. Enzymol.* **155**:335-350.
- Moore, W. E. C. and Lillian V. Holdeman Moore.** 1984. Genus *Eubacterium*, p1353-1370. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's Manual of Systematic Bacteriology*, vol. 2. Williams and Wilkins Co., Baltimore, MD, USA.
- Neefs, J. M., van de Peer, Y., Hendriks, L. and De Wachter, R.** (1990) Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Res.* **18**:2237-2247.
- Newbold, C. J., Wallace, R. J. and McKain, N.** (1990) Effects of the ionophore tetracycline on nitrogen metabolism by ruminal microorganisms *in vitro*. *J. Anim. Sci.* **68**:1103-1109.
- Newbold, C. J., Wallace, R. J. and Watt, N. D.** (1992) Properties of ionophore-resistant *Bacteroides ruminicola* enriched by cultivation in the presence of tetracycline. *J. Appl. Bacteriol.* **72**:65-70.
- Nolan, J. V.** (1975) Quantitative models of nitrogen metabolism in sheep, pp 416-431. In I. W. McDonald and A. C. I. Warner (ed.) *Digestion and Metabolism in Ruminants* University of New England Publishing Unit, Armidale, Australia.
- Nugent, J. H. A. and J. L. Mangan.** (1981) Characteristics of the rumen proteolysis of fraction I (18S) leaf protein from lucerne (*Medicago sativa*). *Br. J. Nutr.* **46**:39-58.
- Odenyo, A. A., Mackie, R. I., Stahl, D. A. and White, B. A.** (1994a) The use of 16S rRNA-targeted oligonucleotide probes to study the competition between ruminal fibrolytic bacteria: development of probes for *Ruminococcus* species and evidence for bacteriocin production. *Appl. Environ. Microbiol.* **60**:3688-3696.
- Odenyo, A. A., Mackie, R. I., Stahl, D. A. and White, B. A.** (1994b) The use of rRNA-targeted oligonucleotide probes to study competition between ruminal fibrolytic bacteria: pure-culture studies with cellulose and alkaline peroxide-treated wheat straw. *Appl. Environ. Microbiol.* **60**:3697-3703.
- Olsen, G. J., Overbeek, R., Larsen, N., Marsh, T. L., McCaughey, M. J., Maciukenas, M. A., Kuan, W.M., Mackie, T.J., Xing, Y. and Woese, C.R.** (1993) The Ribosomal Database Project. *Nucleic Acids Res.* **20**:2199-2200
- Olsen, G. J. and Woese, C. R.** (1993) Ribosomal RNA: a key to phylogeny. *FASEB J.* **7**: 113-123.

Pace, N. R., Stahl, D. A., Lane D. J. and Olsen, G. J. (1985) Analyzing natural microbial populations by rRNA sequences. *ASM News* **51**: 4-12.

Pace, N. R., Stahl, D. A., Lane, D. J. and Olsen, G.J. (1986) The analysis of natural microbial populations by ribosomal RNA sequences. *Adv. Microb. Ecol.* **9**:1-55.

Paster, B. J., Russell, J. B., Yang, C. M. J., Chow, J. M., Woese, C. R. and Tanner, R. (1993) Phylogeny of the ammonia-producing ruminal bacterial *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, and *Clostridium aminophilum* sp nov. *Int. J. Syst. Bacteriol.* **43**:107-110.

Patel, B. K. C., Love, C. A. and Stackebrandt, E. (1992) Helix 6 of the 16S rRNA of the bacterium *Desulfotomaculum australicum* exhibits an unusual structural idiosyncrasy. *Nucleic Acids Res.* **20**:5483.

Piatak, M., Luk, K. C., Williams, B. and Lifson, J. D. (1993) Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. *BioTechniques* **14**:70-80.

Pittman, K. A. and Bryant, M. P. (1964) Peptides and other nitrogen sources for growth of *Bacteroides ruminicola*. *J. Bacteriol.* **88**:401-410.

Pittman, K. A., Lakshmanan, S. and Bryant, M.P. (1967) Oligopeptide uptake by *Bacteroides ruminicola*. *J. Bacteriol.* **93**:1499-1508.

Prins, R. A., Van Gestel, J. C. and Counotte, G. H. M. (1979) Degradation of amino acids and peptides by mixed rumen microorganisms. *Z. Tierphysiol. Tierernahr. Futtermittelk.* **42**:333-339.

Raemakers, L. (1993) Quantitative PCR: theoretical considerations with practical implications. *Anal. Biochem.* **214**:582-585.

Redburn, A. C., and Patel, B. K. C. (1993) Phylogenetic analysis of *Desulfotomaculum thermobenzoicum* using Polymerase Chain Reaction-amplified 16S rRNA-specific DNA. *FEMS Microbiol. Lett.* **113**:81-86.

Reilly, K. and Attwood, G.T. (1998) Detection of *Clostridium proteoclasticum* and closely related strains in the rumen by competitive PCR. *Appl. Environ. Microbiol.* **64**:907-913.

Russell, J. B. (1983) Fermentation of peptides by *Bacteroides ruminicola* B₁₄. *Appl. Environ. Microbiol.* **45**:1566-1574

Russell, J. B. (1987) A proposed model of monensin action in inhibiting rumen bacterial growth: effects on ion flux and proton motive force. *J. Anim. Sci.* **64**:1519-1525.

Russell, J. B. and Baldwin, R. L. (1979) Comparison of maintenance energy expenditures and growth yields among several rumen bacteria grown on continuous culture. *Appl. Environ. Microbiol.* **37**:537-543

Russell, J. B. and Martin, S. A. (1984) Effects of various methane inhibitors on the fermentation of amino acids by mixed rumen microorganisms *in vitro*. *J. Anim. Sci.* **59**:1329-1338.

Russell, J. B., Onodera, R. and Hino, T. (1991) Ruminal protein fermentation: new perspectives on previous contradictions, pp681-697. In Tsuda, T., Sasaki, Y and Kwashima, R., (ed.), *Physiological Aspects of Digestion and Metabolism in Ruminants*, Academic Press, U.K.

Russell, J. B. and Strobel, H. J. (1989) Effect of ionophores on ruminal fermentation. *Appl. Environ. Microbiol.* **55**:1-6.

Russell, J. B., Strobel, H. J. and Chen, G. (1988) Enrichment and isolation of a ruminal bacterium with a very high specific activity of ammonia production. *Appl. Environ. Microbiol.* **54**:872-877.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. F., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487-491.

Saito, H. and Miura, K. I. (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochem. Biophys. Acta* **72**:619-629.

Spencer, D. F., Schnare, M. N. and Gray, M. W. (1984) Pronounced structural similarities between the small sub unit ribosomal RNA genes of wheat mitochondria and *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* **31**:493-497.

Stahl, D. A., Lane, D. J., Olsen, G. J. and Pace, N. R. (1985) Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. *Appl. Environ. Microbiol.* **49**:1379-1384.

Stahl, D. A. and Amann, R. (1991) Development and application of nucleic acid probes, pp 205-248. In Stackebrandt, E., and Goodfellow, M. (ed.), *Nucleic Acid Techniques in Bacterial Systematics*, John Wiley and Sons, Chichester, England.

Stahl, D. A., Flesher, B., Mansfield, H. R. and Montgomery, L. (1998) Use of phylogenetically based probes for studies of ruminal microbial populations. *Appl. Environ. Microbiol.* **54**:1079-1084.

Steffan, R. J. and Atlas, R. M. (1988) DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. *Appl. Environ. Microbiol.* **54**:2185-2191.

Stewart, C. S. and Bryant, M. P. (1988) The rumen bacteria, pp21-75. *In* Hobson, P.N., (ed.), The Rumen Microbial Ecosystem, Elsevier Applied Science, London, U. K.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673-4680.

Van Nevel, C. J. and Demeyer, D. I. (1977) Effect of monensin on rumen metabolism *in vitro*. *Appl. Environ. Microbiol.* **34**:251-257.

Van Gylswyk, N. O. (1990) Enumeration and presumptive identification of some functional groups of bacteria in the rumen of dairy cows fed grass silage-based diets. *FEMS Microbiol. Ecol.* **73**:243-254.

Wallace, R. J. (1979) Effect of ammonia concentration on the composition, hydrolytic activity and nitrogen metabolism of the microbial flora of the rumen. *J. Appl. Bacteriol.* **47**:443-455.

Wallace, R. J. (1986a) Catabolism of amino acids by *Megasphaera elsdenii*. *Appl. Environ. Microbiol.* **51**:1141-1143.

Wallace, R. J. (1992) Acetylation of peptides inhibits their degradation by rumen microorganisms.. *Br. J. Nutr.* **68**:365-372.

Wallace, R. J. (1994) Amino acid and protein synthesis, turnover, and breakdown by rumen microorganisms, pp. 71-111. *In* Asplund, J.M., (ed.) Principles of Protein Metabolism in Ruminants, CRC Press, Boca Raton, FL, USA

Wallace, R. J. (1996) Ruminant microbial metabolism of peptides and amino acids. *J. Nutr.* **126**:1326S-1334S.

Wallace, R. J. and Brammall, M. L. (1985) The role of different species of bacteria in the hydrolysis of protein in the rumen. *J. Gen. Microbiol.* **131**:821-832.

Wallace, R. J., Broderick, G. A. and Brammall, M. L. (1987) Microbial protein and peptide metabolism in rumen fluid from faunated and ciliate-free sheep. *Br. J. Nutr.* **58**:87-93.

Wallace, R. J. and Cotta, M. A. (1988) Metabolism of nitrogen-containing compounds, pp. 217-250. *In* Hobson, P.N., (ed.) The Rumen Microbial Ecosystem, Elsevier Applied Science, London, U. K.

Wallace, R. J., Czerkawski, J. W. and Brekendridge, G. (1981) Effect of monensin on the fermentation of basal rations in the rumen stimulation technique (Rusitec). *Br. J. Nutr.* **46**:131-148.

- Wallace, R. J., Frumholtz, P. P. and Walker, N. D.** (1993a) Breakdown of N-terminally modified peptides and an isopeptide by rumen microorganisms. *Appl. Environ. Microbiol.* **59**:3147-3149.
- Wallace, R. J., McKain, N. and Broderick, G. A.** (1993b) A comparison of methods for determining the concentration of extracellular peptides in rumen fluid of sheep. *Curr. Microbiol.* **26**:333-336.
- Wallace, R. J., Kopecny, J., Broderick, G. A., Walker, N. D., Sichao, L., Newbold, C. J. and McKain, N.** (1995) Cleavage of di- and tripeptides by *Prevotella ruminicola*. *Anaerobe* **1**:335-343.
- Wallace, R. J. and McKain, N.** (1989) Analysis of peptide metabolism by ruminal microorganisms. *Appl. Environ. Microbiol.* **55**:2372-2376.
- Wallace, R. J. and McKain, N.** (1991) A survey of peptidase activity in rumen bacteria. *J. Gen. Microbiol.* **137**:2259-2264.
- Wallace, R. J., Newbold, C. J. and McKain, N.** (1990a) Patterns of peptide metabolisms, pp. 43-50. In Hoshino, S., Onodera, R., Minato, H. & Itabashi, H., (ed.), The Rumen Ecosystem. The Microbial Metabolism and its Regulation. Japan Scientific Societies Press, Tokyo, Japan.
- Wallace, R. J., Newbold, C. J. and McKain, N.** (1990b) Influence of ionophores and energy inhibitors on peptide metabolism by rumen bacteria. *J. Agric. Sci. Camb.* **115**:285-290.
- Wallace, R. J., Onodera, R. and Cotta, M. A** (1997) Metabolism of nitrogen containing compounds, pp. 283-328. In P. N. Hobson and C. S. Stewart (ed.), The Rumen Microbial Ecosystem (2nd edition), Chapman and Hall, London, UK.
- Webb, E. C.** (1992) Enzyme Nomenclature. Academic Press, London, UK.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J.** (1991) 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**:697-703.
- Whetstone, H. D., Davis, C. L. and Bryant, M. P** (1981) Effect of monensin on breakdown of protein by ruminal microorganisms *in vitro*. *J. Anim. Sci.* **53**:803-809.
- Woese, C. R., Fox, G. E., Zablen, L., Uchida, T., Bonen, L., Pechman, K., Lewis, B. J. and Stahl, D. A.** (1975) Conservation of primary structure in 16S ribosomal RNA. *Nature* **254**:83-86.
- Woo, T. H. S., Patel, B. K. C., Smythe, L. D., Symonds, M. L., Norris, M. A. and Dohnt, M. F.** (1997a) Identification of pathogenic *Leptospira* genospecies by continuous monitoring of fluorogenic hybridization probes during rapid cycle PCR. *J. Clin. Microbiol.* **35**:3140-3146.

Woo, T. H. S., Patel, B. K. C., Smythe, L. D., Norris, M. A., Symonds, M.L, and Dohnt, M.F. (1997b) Identification of pathogenic *Leptospira* by *Taq* Man probe in a light cyclor. *Anal. Biochem.* **256**:32-134.

Wright, D. E. (1967) Metabolism of peptides by rumen microorganisms. *Appl. Microbiol.* **15**:547-550.

Wright, D. E. and Hungate, R. E (1967) Amino acid concentrations in rumen fluid. *Appl Microbiol.* **15**:148-151.

Yang, C. M. J. and Russell, J. B. (1993) Effect of monensin on the specific activity of ammonia production by ruminal bacteria and disappearance of amino nitrogen from the rumen. *Appl. Environ. Microbiol.* **59**:3250-3254.

Zachar, V., Thomas, R. A. and Goustin, A. S. (1993) Absolute quantification of target DNA: a simple competitive PCR for efficient analysis of multiple samples. *Nucleic Acids Res.* **21**:2017-2018.

Zheng, D., Alm, E. W., Stahl, D. A. and Raskin, L. (1996) Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. *Appl. Environ. Microbiol.* **62**:4504-4513.